Optimisation of the enzymatic hydrolysis of rainbow trout processing by-products to manufacture liquid fertiliser

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ACRONYMS

AA	Amino Acids
CCD	Central Composite Design
CT1	Combination Treatment 1
CT2	Combination Treatment 2
CT3	Combination Treatment 3
DH	Degree of Hydrolysis
E/S	Enzyme/Substrate ratio (% w/w of substrate concentration)
FAA	Free Amino Acids
FPH	Fish Protein Hydrolysate
LAB	Lactic acid Bacteria
NPK	Nitrogen, Phosphorous and Potassium
OPA	O-phthaldialdehyde
ROS	Reactive Oxygen species

ABSTRACT

Fish processing by-products are a potential source of proteins for a variety of applications, amongst them fertilisers. Since these by-products are otherwise dumped in landfills, resulting in environmental contamination, their recovery presents an economic opportunity to derive value from these otherwise discarded materials. There exist two main technologies for fish fertiliser production; fish emulsion and fish protein hydrolysates (FPH). Fish fertilisers contain both macro (nitrogen, phosphorous, potassium, calcium and magnesium) and micro (iron, manganese, zinc, copper, molybdenum, and boron) nutrients, with the ability to provide nutrients to both the plants and the soil micro-organisms, thereby building a healthy soil profile. Depending on the characteristics, fish fertilisers can be applied both as soil additives and as foliar spray.

South Africa produced 1800 metric tonnes of rainbow trout in 2008, of which a significant amount ends up as by-products after processing. Such a supply of rainbow trout by-products is substantial to sustain the protein recovery processes at a commercial scale. This work is an investigation into the production of a liquid fertiliser, suitable both as a foliar and soil application, using rainbow trout processing by-products (heads) as raw material.

The work was divided into three stages. The first stage involved determining the optimum hydrolysis conditions for two pre-selected proteolytic enzymes; SEBPro XL, an exoprotease and SEBDigest F59P, an endoprotease. To determine optimal conditions, three independent variables namely temperature, pH and E/S ratio were optimised at five levels ($-\alpha$, -1, 0, $+1$, and $+\alpha$) by using response surface methodology, central composite design. The degree of hydrolysis, which is the extent of the hydrolysis process, was the dependent variable. The DH was measured and monitored according to the modified spectrophotometric o-phthaldialdehyde (OPA) method. Optimal hydrolysis conditions for optimum DH were found to be a temperature of 60^{o}C for both enzymes. The optimum pH for SEBPro XL was 6.9 whilst for SEBDigest F59P, the optimum pH was 7.6. Within the employed experimental domain, the optimum temperatures and pH were determined at an enzyme to substrate ratio (E/S) of 0.05% and 0.67% for SEBDigest F59P and SEBPro XL respectively. The E/S ratios were based on the protein content of the by-product fish heads.

The second stage involved hydrolysing with the two enzymes at their optimum conditions for three hours. The main purpose of this stage was to maximise the DH using the two enzymes and their combinations. With three combinations of the two enzymes being designed, a total of five enzyme treatments were considered for the hydrolysis experiments. These five enzyme treatments were employed in the hydrolysis process and their fish protein hydrolysates (FPH) were compared by way of DH, amino acid (AA) content, free amino acid (FAA) content, macro and micro mineral content of the fertiliser, as well as the heavy metals. The results showed that using SEBPro XL alone results in higher levels of total AA and a higher proportion of FAA in the FPH compared to all other treatments. Its DH was also higher than that of all other treatments. All enzyme treatments produced FPH of within limits heavy metals as per the requirements of the fertiliser regulations and significant amounts of macro and micro nutrients.

In the third and final stage of the investigation, SEBPro XL was further employed at varying E/S ratios to maximise on the DH and shorten the reaction time. The E/S ratios ranged from 1% to 5%. The reactions were run for four hours each. At the end of hydrolysis, the DH was 38% for a 1% ratio, 43.1% for 2% ratio, 50.9% for 3% ratio, 58.2% for 4% ratio and 60.2% for a 5% E/S ratio. Therefore depending on the desired DH, a choice can be made for an enzyme concentration and time of hydrolysis.

This work established the optimum temperature and pH for hydrolysing rainbow trout processing by-products heads with two enzymes, SEBPro XL and SEBDigest F59P. It also demonstrated the possibility of producing foliar fertiliser from rainbow trout processing by-products. The fertiliser's nutrient content lies within the legislative constraints. By manipulation of the reaction conditions, temperature, pH and enzyme concentration, as well as reaction time, its quality may be pre-determined.

OPSOMMING

Verwerkte by-produkte van vis is 'n potensiële bron van proteïene vir 'n verskeidenheid van toepassings, onder andere soos kunsmis. Sedert hierdie by-produkte in stortingsterreine gestort word, kan dit lei tot omgewing besoedeling waar hierdie herstel ’n ekonomiese geleentheid verteenwoordig om waarde uit hierdie weggooi materiaal af te lei. Daar bestaan twee hoofsaaklike tegnologieë vir die kunsmis produksie van vis nl. vis emulsie en vis proteïen hidrolisate (FPH). Vis kunsmis bevat beide makro- (stikstof, fosfor, kalium, kalsium en magnesium) en mikro- (yster, mangaan, sink, koper, molibdeen, en boor) voedingstowwe, wat die vermoë het om voedingstowwe aan beide plante en die grond mikro-organismes te voorsien asook om ’n gesonde grondprofiel te bou. Afhangende van die karaktereienskappe kan vis kunsmis toegepas word beide grondbymiddels en as blaar besproeiing.

Suid-Afrika het 1800 metrieke ton reënboog forel in 2008 geproduseer waarvan 'n beduidende hoeveelheid beland het as by-produkte na die verwerking daar van. So aansienlike aanbieding van reënboog forel by-produkte word gebruik om die proteïen herstel prosesse, teen 'n kommersiële skaal in stand te hou. Hierdie werk dien as 'n ondersoek in die vervaardiging van 'n vloeibare kunsmis tipe wat geskik sal wees vir beide blaarbespuiting en grond toediening wat met die hulp van reënboog forel verwerkte by-produkte (koppe) as rou materiaal, sal dien.

Die werk word in drie fases gedeel. Die eerste fase is betrokke by die bepaling van die optimum hidroliese voorwaardes vir twee geselekteerde ensieme SEBPro XL ('n exoprotease) en SEBDigest F59P ('n endoprotease). Om optimale omstandighede te bereken, sal drie onafhanklike veranderlikes nl. die temperatuur, pH en ensiemkonsentrasie optimiseer word teen vyf vlakke (-α, -1, 0, 1, en + α), met behulp van die reaksie oppervlak metode, sentrale saamgestelde ontwerp. Die graad van hidrolise, was die afhanklike by die omvang van die hidroliese proses. Die DH is gemeet en gemonitor volgens die aangepaste spektrofotometries o-phthaldialdehyde (OPA). Die optimale hidroliese omstandighede vir die optimale DH was gevind teen 'n temperatuur van 60^{o}C vir beide ensieme. Die optimale pH vir SEBPro XL was 6,9 terwyl SEBDigest F59P se optimale pH was 7.6 was. Binne die perke van die eksperimentele domein, is die optimaal temperature en pH bepaal by 'n ensiem die substrate verhouding (E/S) van 0,05%, 0,67% vir SEBDigest F59P en SEBPro

XL onderskeidelik te bepaal. Die E/S verhoudings was gebaseer op die proteïeninhoud van die by-produk viskoppe.

Die tweede fase is betrokke met die hidrolisering van twee ensieme by hul optimale vir twee ure lank. Die hoofdoel van hierdie fase was om die DH te maksimeer deur gebruik te maak van twee ensieme en hul kombinasies. Met drie kombinasies van die twee ensieme wat ontwerp is, was 'n totaal van vyf ensieme behandelings in aanmerking gekom vir die hidroliese eksperimente. Hierdie vyf ensiem behandelings was in diens gestel in die hidroliese proses en hul vis proteïen hidrolisate (FPH) was vergelyk deur middel van DH, aminosuur (AA) inhoud, bevry aminosuur (FAA) inhoud, makro en mikro minerale inhoud van die kunsmis sowel as die swaar metale. In vergelyking met die ander behandelinge, het die resultate getoon dat die allenige gebruik van die SEBPro XL hoër vlakke van totale AA getoon het asook 'n hoër proporsie van FAA in FPH. Sy DH was ook hoër as dié van al die ander behandelings. Alle ensiem behandelings produseer FPH binne die perke van swaar metale soos uiteengesit deur die kunsmis regulasies vereistes asook die aansienlike hoeveelhede van makro en mikro voedingstowwe.

In die derde en finale fase van die ondersoek, is SEBPro XL verder in diens gestel teen wisselende E/S verhoudings om die DH te maksimeer en die reaksie tyd te verkort. Die E/S verhoudinge wissel van 1% tot 5%. Die reaksies is gehardloop vir vier ure elk. Aan die einde van hidrolise, was die DH 38% vir 'n 1% verhouding, 43.1% vir ’n 2% verhouding, 50.9% vir ’n 3% verhouding, 58.2% vir ’n 4% verhouding en laastens 60.2% vir ’n 5% verhouding. Afhangende van die gewenste DH, kan 'n keuse gemaak word vir 'n ensiem konsentrasie en tyd van hidrolise.

Hierdie werk het die optimale temperatuur en pH vir die hidrolisering van verwerkte reënboog forel by-produkte gevestig deur middel van twee ensieme nl. SEBPro XL en SEBDigest F59P. Dit het ook die moontlikheid om blaar voedingstowwe te vervaardig van reënboog forel by-produkte, gedemonstreer. Die kunsmis se voedingstowwe lê binne die wetgewende beperkings. Deur die reaksie kondisies, temperatuur, pH en ensiem konsentrasie asook reaksie tyd te manipuleer, kan die kwaliteit vooraf bepaal word.

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1. INTRODUCTION

1.1 BACKGROUND

Enzymatic protein hydrolysis has been shown to be an effective method for the recovery of essential nutrients from fish processing by-products (Himonides *et al.*, 2011; Nguyen *et al.*, 2010; Nilsang *et al.*, 2004; Opheim *et al.*, 2015; Ovissipour *et al.*, 2010; Turid, 2003). During protein hydrolysis, proteolytic enzymes hydrolyse peptide bonds in proteins, resulting in a mixed product consisting of proteins, peptides and free amino acids. Enzymatic hydrolysis can be achieved by the application of either endogenous or exogenous enzymes (Bhaskar *et al.*, 2008). The application of endogenous enzymes (autolysis) was traditionally used to prepare autolytic hydrolysates like fish silage. However the method presents difficulties in process control, due to the different types and amounts of enzymes present in the fish which change with different factors like seasonality, fish species, sexual maturity, etc (Sikoroski & Naczk, 1981). By employing exogenous enzymes, the hydrolysis process is made controllable, faster and reproducible (Je *et al.*, 2004). Several factors like pH, time, enzyme to substrate ratio (E/S) and temperature, significantly affect the activities of enzymes co-operatively and thus, by manipulation of these factors, the hydrolysis can be controlled (Liaset *et al.*, 2000; Viera *et al.*, 1995). Subject to the specificity of the enzyme and the extent of hydrolysis, measured by the degree of hydrolysis (Bhaskar *et al.*, 2008), peptides of varying sizes are generated, which will determine the particular properties and function of the resultant fish protein hydrolysate (FPH) (Kechaou *et al.*, 2013).

South Africa's rainbow trout (*Oncorhynchus mykiss*) production from aquaculture reached 1800 metric tonnes in 2008 (Stander, 2009), and currently significant expansion of the sector is under way in Lesotho as a result of the Lesotho Highlands Water Project (Royale Highlands Trout). Since market preferences vary, trout is processed and sold in a variety of value added products, including whole gilled and gutted, smoked fish and fillets, as well as live fish for supplying the sport fishery sector (Stander, 2009; Stander *et al.*, 2011). The processing releases frames, heads, fins, tails, skins and guts as by-products (Ghaly *et al.*, 2013). These by-products contain valuable proteins (Murna Muzaifa *et al.*, 2012) that will present an economic loss if left unrecovered. The by-products are also environmentally damaging (Christie & Moldan, 1977), yet useful essential

nutrients e.g. essential amino acids and lipids, can be recovered if they are to be utilised (Aasen, 2014).

Apart from macronutrients like proteins and lipids, fish processing by-products have been confirmed to contain compounds like nucleic acids, organic acids, vitamins and sugars (Iizuka & Konishi, 1995). These compounds are capable of promoting plant growth (Day & Katterman, 1992). Fish processing by-products also differ from other process wastes like some municipal and industrial effluents in that they do not contain known toxic materials (Afonso & Bórquez, 2002). Therefore the fish protein hydrolysates could be valuable resources as organic liquid fertilisers, with an added benefit of environmental friendliness. Studies on the recovery of fish processing by-products to manufacture liquid fertiliser have been conducted recently by different researchers (Beckley & Barrie, 2010; Iizuka & Konishi, 1995; Milnes, 2015).

According to a study done by Joong Kyun Kim (Kim, 2011), small scale conversion of fish processing by-products into liquid fertiliser is currently not economically attractive. However, commercial production at a large scale could reduce operation costs and bring benefits of economies of scale. Therefore for economic viability of converting fish processing by-products to liquid fertiliser, large scale industrial applications of optimised processing operations should be considered. Optimisation of the enzymatic hydrolysis of fish processing by-products was successfully done by different researchers (Bhaskar *et al.*, 2008; Kechaou *et al.*, 2013; Nilsang *et al.*, 2004; Ovissipour *et al.*, 2010) using a variety of combinations of fish species and enzymes.

This study was undertaken to optimise the enzymatic hydrolysis of rainbow trout processing by-products to manufacture an FPH applicable as a foliar spray. Since enzymatic protein hydrolysis processes have different reaction rates depending on temperature and pH, it is necessary that these factors be set at their optimum levels to achieve the highest possible DH (at least 45%) and free amino acid content in the resultant FPH. Further, a suitable E/S ratio also needs to be determined in order to have sufficient rate of hydrolysis, without incurring unacceptably high enzyme costs. A high DH translates to a high concentration of simpler peptides and free amino acids in solution (Opheim *et al.*, 2015; Panyam & Kilara, 1996). A high DH is therefore desirable since the simpler peptides and free amino acids released can be absorbed through plant leaves and roots to provide ready-made blocks for protein synthesis in plants.

The possibility of establishing a relationship amongst the DH, E/S and time was also investigated. Such a relationship would define how to increase the expected DH under specific conditions of time and E/S. If such a relationship could be established, it would be possible for specific E/S and time to be set with respect to the required DH, therefore making enzymatic hydrolysis predictable in an industrial set up.

1.2 PROBLEM STATEMENT AND MOTIVATION

1.2.1 Problem in context

An industrial company intends to open a fish protein hydrolysis fertiliser manufacturing plant. The hydrolysis will be achieved enzymatically and two preliminary enzymes have been selected from the wide range of commercially available candidates, based on availability and preliminary supplier data. Enzymes are sensitive to pH and temperature, as well as the presence of inhibitors and denaturants (Mótyán *et al.*, 2013; Robinson, 2015), and only catalyse reactions within a specific pH and temperature range, therefore, the hydrolysis reaction must be carefully monitored and controlled. Whereas the operating temperature and pH ranges for the two enzymes may be available in literature, optimum operating conditions are substrate specific and they were unknown for rainbow trout by-products.

It was not known whether the pre-selected enzymes would produce optimal results when employed individually in a reaction, or when used in combination. The enzymes, one being an endoprotease and the other exoprotease, are expected to complement each other - the endoprotease digesting proteins into poly-peptides whilst the exoprotease produces simpler peptides and free amino acid (Advanced Enzymes Technologies). It was this study's obligation to ascertain whether the enzymes will best be applied individually or in combinations. The actual combinations were designed and tested

Due to minimum product requirements for the particular application, a DH value of at least 45% has been specified by the industry. Furthermore, due to operational considerations, a maximum hydrolysis time of 4 hours was specified as an additional constraint which will allow for the actual hydrolysis and other processes (including mincing, filtration and packaging) to fit into an eight hour shift, which is currently applicable in the concerned industrial set up. It was also the other purpose of this study to establish whether such a DH is attainable in the time constraint given, and define the conditions under which it will be satisfied.

The key research questions of the study were:

i. Will the fish protein hydrolysate resulting from the enzymatic hydrolysis of rainbow trout processing by-products be suitable as a foliar, organic fertiliser?

ii. What are the optimum operating conditions (i.e. pH, temperature and enzyme concentrations) for each of the two pre-selected enzymes?

iii. Which of the two available enzymes, or their possible combination treatments, will be most suitable to produce the best fertiliser with regards to DH and FAA?

iv. Which hydrolysis conditions, if possible, will result in a DH of at least 45% within 4 hours of hydrolysis?

v. Will the resultant DH of the fish protein hydrolysate be predicted from the enzyme concentration and reaction time when hydrolysing at optimum conditions?

Problem statement

A new fish protein hydrolysate factory is being setup to produce FPH fertiliser and there is need to optimise processing conditions in order to produce a consistently high-quality product, within specifications as set out by the client (at least 45% DH) within four hours of hydrolysis time.

1.3 RESEARCH OBJECTIVES

The aim of this project was to develop an enzymatic hydrolysis process for the production of fish protein hydrolysate from rainbow trout by-products, for use as a liquid fertiliser.
The specific objectives for the project were as follows;

i. To determine the optimum hydrolysis conditions of the two candidate proteolytic enzymes, using a structured statistical approach.

ii. To hydrolyse rainbow trout processing by-products separately with the two candidate enzymes and their possible combinations and evaluate the characteristics (DH and FAA) inherent to each enzyme treatment.

iii. To select the best performing enzyme treatment with regards to DH and FAA.

iv. To maximise the DH to at least 45% and a high FAA content by increasing the E/S ratio from 1 to 5% of the by-product protein content.

1.4 SIGNIFICANCE OF STUDY

This study was sponsored by industry to specify the optimal conditions for the hydrolysis of rainbow trout processing by-products. It is therefore going to be a vital reference tool for setting up and operation of the facility. It is also a significant contribution towards creating value from an underutilised resource (trout processing by-products), whilst at the same time addressing a significant and immediate industrial need.

Since enzymes are affected by pH, temperature and E/S ratio, it is important that the optimal values of these parameters are observed for every hydrolysis reaction. Enzymes are also specific, therefore behaviour of each combination of enzyme and substrate are expected to be unique. Various authors have reported different optimised conditions for different enzymes and species, Mahmoudreza Ovissipour (Ovissipour *et al.*, 2010) on yellowfin tuna, Kechaou and colleagues (Kechaou *et al.*, 2013) on common cuttlefish and Guerard and colleagues (Guerard *et al.*, 2001) on yellowfin tuna. The research completed in this investigation focused on a fish species called rainbow trout (*Oncorhynchus mykiss*) and two enzymes, SEBPro XL and SEBDigest F59P were employed. This information is not reported in literature, and therefore the result generated in this study adds to the database of optimisation of enzymatic hydrolysis of fish processing by-products.

2. LITERATURE REVIEW

2.1 FISH BY-PRODUCTS APPLICATIONS

Fish processing by-products include heads, tails, skins, guts, fins and frames. They have potential to be sources of proteins, collagen and gelatine, amino acids, oils, enzymes and other value added products if recovered and reprocessed (Ghaly *et al.*, 2013; Kristinsson & Rasco, 2000; Logesh *et al.*, 2012; Pasupuleti & Braun, 2010). It is therefore plausible that inedible fish processing by-products could be utilised rather than disposed of. Development of value adding technologies have also identified bioactive compounds which could be extracted from marine processing by-product materials, bringing more value from what has been considered a waste (Arason *et al.*, 2009). A brief description of some current uses of fish processing by-products is given below.

The possible applications for the fish processing by-products are determined by the laws and regulations that govern by-product collection, transportation, storage, handling, processing and disposal. For instance, according to the European Union (EU) legislation, materials that are unfit for human consumption are classified as animal by products (ABP) and can only be processed at appropriate plants (Aspevik *et al.*, 2017).

2.1.1 Production of fish meal

One of the major methods of processing fish by-products, is the production of fishmeal. Fishmeal is a dry powder prepared from whole fish or from fish processing by-products, for use as feed for aquaculture and livestock (Beckley & Barrie, 2010; Chamberlain, 2011), and as high quality organic fertiliser (Abowei & Tawari, 2011). In South Africa, the largest consumer of fish meal is the poultry industry (Koning, 2005).

Although there are many processes of producing fish meal from processing by-products, two are mostly widely used, the wet and dry processes (Abowei & Tawari, 2011). Choosing between the two processes depends on the oil content of the fish by-products concerned (Pigott, 1967). For raw material with an oil content greater than 2.5-3%, the wet process is used since it reduces the oil content in the fish meal to within acceptable levels (Abowei & Tawari, 2011; Pigott, 1967). The wet process produces two products, fish oil and fish meal. The method is a six step procedure. In

the first step, the raw material is heated to a temperature of 95-100ºC (FAO, 1986) and the protein present is deformed, liberating oil and water. The resultant broth is then subjected to pressure to yield press cake and a mixture of oil and water. The oil is separated from the water phase (stick water) whilst the press cake is broken into small pieces in a hammer mill and dried. The stick water is subsequently concentrated to a viscous liquid and added back to the press cake prior to the final drying process (Barlow & Windsor, 1984). The oil fraction separated from the stick water undergoes a series of refining steps dictated by the intended end use. One such a refining step for oil used for human consumption is polishing. This is the stripping of oil by steam or hot water to remove moisture and residual meal components which may cause oil degradation (FAO, 1986; Pigott, 1967).

The dry process is used to manufacture fish meal from lean (oil content less than 2.5%) fish processing by-products (Abowei & Tawari, 2011). The process involves grinding the fish processing by-products and a combined cooking and drying step, with the pressing step eliminated (Pigott, 1967). There is no fish oil produced in this process.

Fish meal inclusion in poultry and chicken feeds is highly regarded due to its high level of lysine, the unique combination of essential amino acids and trace metals, the sulphur-containing amino acids as well as unidentified growth factors (UGFs) (Karrick, 1963; Pike, 1975). It is however not economically viable where relatively small amounts of by-products are produced (Abowei & Tawari, 2011). As a result, fish processing by-products are still discarded in some instances (Blanco *et al.*, 2007; FAO, 1986).

2.1.2 Production of fish oil

Fish oil can be produced from fish processing by-products enzymatically, chemically, or by cooking and pressing (Ghaly *et al.*, 2013; Ramakrishnan *et al.*, 2013). The cooking and pressing method also produces fish meal (refer to section 2.1.1). The chemical processes include Soxhlet or Goldfisch method (Hwang & Regenstein, 1988), Chloroform/ Methanol method (Folch *et al.*, 1957), Bligh and Dryer method (Bligh & Dyer, 1959) using solvents like diethyl ether, petroleum ether, methanol and hexane, as well as acid digestion method with hydrochloric acid (Radar, 1995; Shahidi, 2003). Concerns about the chemical extraction methods are that the methods are complicated, requiring excess energy for recovery of the solvents used for extraction. The methods

also produce environmentally unfriendly by-products. The cooking and pressing method is also very harsh on lipids, with potential to quicken their degradation and therefore loss of nutritional value (Dauksas *et al.*, 2005).

Enzymatic hydrolysis for production of fish oil is more favourable than the other methods because it is a low temperature method and produces no waste by-product (Ramakrishnan *et al.*, 2013). It also increases the oil extraction yield and produces oils with higher concentrations of omega -3 fatty acids (Linder *et al.*, 2005; Slizyte *et al.*, 2005). In the enzymatic extraction method, the fish by-products are minced and mixed with water or a buffer in equal proportions. The hydrolysis conditions (temperature and pH) of the slurry mixture are then adjusted to optimal values depending on the employed enzyme and the substrate, after which the hydrolysis is started by adding the enzyme to the mixture. The hydrolysis proceeds for a set time period and then heated at 90°C for 5 min to deactivate the added enzymes. The mixture is then centrifuged to separate the oil fraction (Gbogouri *et al.*, 2004; Hathwar *et al.*, 2011; Slizyte *et al.*, 2005).

Fish oils can be used for human consumption, as ingredients for animal feed or for biodiesel production, the latter being produced from waste, poor quality oil (Ghaly *et al.*, 2013; Zuta *et al.*, 2003). When used for human consumption and animal feed, fish oils are valuable as good sources of long chain polyunsaturated fatty acids (PUFA) (omega-3 fatty acids), which consist primarily of eicosapentaenoic acid (Department of Agriculture 2012) and docosahexaenoic acid (Bhaskar *et al.*, 2008; Ibanez *et al.*, 2012; Khoddami *et al.*, 2009). These omega-3 fatty acids are highly valuable due to their bioactivities, which include prevention of cardiovascular diseases, reduced blood pressure, protection against pulmonary diseases, and improved learning ability, among others (Kim & Mendis, 2005; Sahena *et al.*, 2009; Wu & Bechtel, 2008; Zuliani *et al.*, 2009).

2.1.3 Bioactive compounds

Fish processing by-products contain polysaccharides, polypeptides, amino acids and fatty acids with bioactive properties (Kim & Mendis, 2005; Trung & Phuong, 2012). These substances, called bioactive compounds may have antitumor, antioxidant, antithrombotic, anti-inflammatory, immune modulatory, anti-hypertensive and antimicrobial functional properties (Kim *et al.*, 2000). Bioactive compounds are defined as food derived components that, in addition to their nutritional value, exert a physiological effect in the body (Vermeirssen *et al.*, 2007).

The bioactive compounds are ineffective, until they are liberated from the parent substrate, mostly through enzymatic hydrolysis (Arihara *et al.*, 2001; Jang *et al.*, 2008; Katayama *et al.*, 2008; Liu *et al.*, 2008). After enzymatic hydrolysis of the parent substrate, the compounds are screened according to solubility, heat resistance, or molecular weight and the bioactivity that is sought (Ibanez *et al.*, 2012). One proven way of performing the screening is by ultrafiltration (Iroyukifujita *et al.*, 2000; Ondetti *et al.*, 1977) and then further purification of the highest bioactive fraction to separate individual compounds. This step is mostly done using reverse phase high performance liquid chromatography (RP-HPLC) or gel permeation chromatography (Saiga *et al.*, 2006, 2008). There are also other alternative ways to extract bioactive compounds, like the solid-liquid extraction, Soxhlet and the ultrasound-assisted extraction methods but they have become unfavourable, mostly due to environmental concerns, high costs and lower efficiency (Baiano, 2014). Microbial fermentation was also not successful because the lactobacilli used in the protein fermentations has poor activity (Arihara & Ohata, 2006; Hammes *et al.*, 2003).

2.1.4 Collagen and gelatine

Collagen is the dominant fibrous protein in connective tissue which is found in various forms in tissues of all species of multicellular organisms (Schmidt *et al.*, 2016). The main sources of collagen are the skin, tendons, cartilage and bones and it constitutes 30% of fish bone (Logesh *et al.*, 2012). Collagen extraction can be achieved by treating the fish by-products with hot low strength acid or by using salt solution. It is then denatured, through hydrolysis catalysed either by enzymes, acid or alkali, in order to produce a soluble gelatine product (Ran & Wang, 2014; Yu *et al.*, 2014). The conversion of collagen to gelatine breaks the inter-and intra-molecular bonds linking collagen chains, as well as some peptide bonds, disrupting the collagen native protein structure (Cole & McGill, 1988; Zhou *et al.*, 2006).

Gelatine finds numerous applications in the food, cosmetics and biomedical industries due to its protein content and functional properties, such as water absorption capacity, gel formation, and the ability to form and stabilise emulsions (Lafarga & Hayes, 2014; Schmidt *et al.*, 2016). In the biomedical and pharmaceutical fields for example, gelatine is used as a vehicle for drugs, proteins

and genes, as well as a substitute for human skin, blood vessels and ligaments (Gómez-Guillén *et al.*, 2011; Kim & Mendis, 2005).

2.1.5 Fertilisers

Conventionally, soil additives and plant fertilisers have been made synthetically from petroleum by-products and mineral salts. However, increasing public awareness of the environmental drawbacks of using chemical-based fertilisers has created a demand for safe, natural and more environmentally friendly fertilisers (Beckley & Barrie, 2010). Fish and their processing by-products (frames, blood, heads and guts) are a rich source of protein, which can be processed to produce an environmentally friendly organic fertiliser (Benjakul & Morrissey, 1997; Iizuka & Konishi, 1995). Such components as organic nitrogen (which contains amino acids, such as lysine, asparagine, glutamine, serine, tyrosine, proline, etc.), nucleic acids, vitamins and sugars are a good basis of organic fish fertilisers (Iizuka & Konishi, 1995). Fish protein hydrolysis fertilisers can be profitably produced commercially, and are also a viable alternative to non-renewable petroleum products, as they are made from a renewable resource (Beckley & Barrie, 2010).

2.1.5.1 FPH as fertiliser

Traditionally fish by-products were spread on the ground, or buried in the soil to fertilise crops (Archer *et al.*, 2001; Goode, 1880). However, by hydrolysing the fish processing by-products, the handling and application of the fish fertiliser are made easier. The FPH produced can also be concentrated to reduce its bulkiness, thus cutting down on storage and transportation costs (Benjakul & Morrissey, 1997; Bhaskar *et al.*, 2008; Pasupuleti & Braun, 2010). Acceptability is generally higher in countries like the United States of America and New Zealand for FPH fertilisers with companies like BioMarinus, Safer Grow, Sea Grow, Humofert and Neptune's Harvest manufacturing and supplying FPH fertiliser on a commercial scale.

Plants can absorb free amino acids through their roots and leaves (King, 2010; Milnes, 2015; Quan *et al.*, 2012; Wolf-Nicolas Fischer *et al.*, 1998). This ability of plants to absorb free amino acids through their roots and leaves makes fish protein hydrolysis fertiliser applicable and functional as a foliar and soil additive. Amino acids are well known to increase crop yield and quality, as well as senescence retardation (Sadak *et al.*, 2015). Their application for foliar use might be for general

plant fertiliser requirements or critical for stress mitigation (Hayat *et al.*, 2012b; Rai, 2002; Szabados & Savoure, 2009). When applied as a soil additive, the fish protein hydrolysis fertiliser feeds soil organisms, improving the soil microbial activity and ultimately building a good soil structure (Milnes, 2015).

Effect of free amino acids on plants

Accumulated free amino acids in plants, besides functioning as the building blocks of proteins, influence a number of physiological processes such as plant growth and development, intracellular pH control, generation of metabolic energy or redox power, and resistance to both abiotic and biotic stress, as well as regulation of ion transport, detoxification of heavy metals and gene expression. Some of the free amino acids functions in plants are as follows;

- **Stress resistance**

The environment sometimes subjects plants to various types of environmental stresses which include salinity, water deficit, temperature extremes, toxic metal ion concentration and UV radiation. These conditions affect negatively the plant metabolic processes, depending on severity of the stress (Hayat *et al.*, 2012b). Applying exogenous free amino acids before the stress is a preventative measure, whilst applying during and after the stress conditions presents mitigating and recovering effects. There are some amino acids which are directly related to stress physiology, like proline, which mitigates water stress, as well as alanine, serine and asparagine (Rai, 2002).

- **Stomatal regulation**

Stomas are the cellular structures responsible for controlling the hydric balance of the plant, the macro and micronutrient absorption and the absorption of gases. They are alternatively opened and closed depending on this balance (Sharma & Rai, 1989). Their opening is a direct function of both external factors (light, humidity, temperature and salt concentration) and internal factors (amino acids concentration, abscisic acid etc.) They are closed when light and humidity are low and temperature and salt concentration are high. At the closed position, photosynthesis and transpiration are reduced and respiration is increased. This causes a negative metabolic balance of the plant. Rai and Sharma (Rai & Sharma, 1991) showed that in *Vicia faba* leaf epidermal peels, proline, glycine, alanine, leucine, threonine, lysine, arginine, tryptophan and phenylalanine

inhibited stomatal opening while histidine, methionine, aspartic acid, glutamic acid, asparagine and glutamine promoted stomatal opening.

- **Chelating Effect**

Free amino acids applied as soil additives can have an important role in regulating metal toxicity in plants (Rai, 2002). Work by Sharma (Sharma *et al.*, 1998) showed that proline protected the activity of glucose-6-phosphate dehydrogenase and nitrate reductase against inhibition by cadmium and zinc, due to formation of a proline -metal complex. Farago and Mullen (Farago & Mullen, 1979) showed an existence of a Cu-proline complex in a metal tolerant *armeria.* Work by Krammer and colleagues (Krammer *et al.*, 1996) reported an increase of histidine in *alyssum* that was proportional to applied nickel. They showed a Ni-histidine complex in the xylem sap. When applied together with chemical fertiliser, free amino acids in FPH fertiliser improve nutrients availability by chelating them and rendering them more soluble for uptake up the plant roots (King, 2010).

- **Proline**

Proline has been singled out as the most influential amino acid, playing critical roles in controlling both abiotic and biotic stresses and ensuring good plant health (Szabados & Savoure, 2009). The accumulation of proline in distressed plants has been reported by many researchers (Hayat *et al.*, 2012b; Rai, 2002; Rai & Sharma, 1991). In their reviews on proline accumulation during various plant stresses, Hayat and co-workers (Hayat *et al.*, 2012a), found that exogenous proline plays three key roles in combating plant stresses, as a metal chelator, an anti-oxidative defence molecule and a signalling molecule. Rai also reviewed the role of amino acids and confirmed the multi-functionality of exogenous proline (Rai, 2002). The roles of proline can be summarised as follows;

a. It protects the plants from various stresses and also helps plants to recover from stress more rapidly.

b. When applied exogenously to plants exposed to stress, proline results in enhanced growth and other physiological characteristics of plants.

c. Exogenous proline scavenges the reactive oxygen species (Sikoroski & Naczk, 1981) generated in plants under various biotic and abiotic stresses.

d. Exogenous proline application affects plant-water relations by maintaining turgidity of cells under stress, and also increases the rate of photosynthesis.

e. Exogenous proline application to plants protects them from harmful radiation such as UV-B.

f. Low concentrations of exogenous proline protect plants from salinity, drought and temperature stress. However, higher doses will impart toxic effects.

- **Tryptophan**

Tryptophan (Trp) is one of the three aromatic amino acids (the other two are tyrosine and phenylalanine) which are synthesised through the *Shikimate* pathway in plants (Dewick, 2002). These aromatic amino acids are the precursors for a large variety of secondary metabolites with aromatic ring structures that often make up a substantial amount of the total dry weight of a plant. Among the many aromatic secondary metabolites are flavonoids, many phytoalexins, indole acetate, alkaloids such as morphine, ultra violet light protectants, and most importantly, lignin (Herrmann, 1995). Mutations that disrupt Trp biosynthesis result in various developmental defects in plant organs. Plants also use Trp as a precursor to produce indole and anthranilate derived alkaloids, which play direct roles in regulating plant development, pathogen defence response and plant–insect interaction (Hildebrandt *et al.*, 2015; Radwanski & Last, 1995; Rai, 2002; Woodward & Bartel, 2005). Tryptophan is essential for the synthesis of auxins (such as indole-3-acetic acid), which are important growth hormones, methionine is a precursor of ethylene, an important plant hormone implicated in development and stress signalling whereas isoleucine is necessary for the activation of jasmonic acid (Kadotani *et al.*, 2016).

2.1.5.2 Comparing FPH fertiliser to conventional fertiliser

FPH fertilisers contain significant quantities of organic, protein nitrogen as well as a healthy balance of all 18 nutrients and amino acids which are significant for plant health and growth (Beckley & Barrie, 2010). They are able to replenish the nutrient level of the soil and feed important soil organisms, such as nematodes, earthworms and microorganisms, increasing the bacterial and fungal activities in the soil and creating a well aerated and healthy soil structure (Lema & Degebassa, 2013). Chemical fertilizers do not contain as much micro and macronutrients as FPH fertilisers and do not contain organic nitrogen for microbes to feed on. As such, chemical fertilisers can only treat plants but not the soil (Beckley & Barrie, 2010; Milnes, 2015). This results in degraded soil structures that will require high chemical fertiliser applications oftenly to maintain crop quality and growth (Magdoff & Weil, 2004).

Liquid FPH fertiliser when applied as foliar spray (Hildebrandt *et al.*, 2015; Szabados & Savoure, 2009; Wolf-Nicolas Fischer *et al.*, 1998), is a quick and effective way of providing a solution to nutrient imbalance as they are speedily absorbed through the leaves.

FPH fertilisers do not leach from the soil as much as synthetic fertilisers, so their contribution to water pollution is minimal (Milnes, 2015). Chemical fertilisers leach nitrates, phosphates and urea into the ground water if applied abundantly and are not immediately taken up by plants (Harman & Lela, 2014). According to Arnall (Arnall, 2009), plants typically use only about 33% of the total nitrogen fertilizer applied while the remainder may be metabolised to nitrous oxides that are potent greenhouse gases or leach into soil and surface water as nitrates and nitrites, where they may be toxic and result in eutrophication of water bodies. FPH fertilisers, like other organic fertilisers, are environmental friendly, do not leach readily and stay long in the soil where they provide plant nutrition for longer durations than chemical fertilisers (Beckley & Barrie, 2010).

2.1.5.3 Nutrients needed for plant growth

There are a total of sixteen essential elements for plant growth, which are sourced from the soil solution and the atmosphere (FAO, 2000). The element carbon comes from carbon dioxide (CO_2) in the atmosphere. Water (H_2O) provides hydrogen and oxygen, whilst the rest of the nutrients come from fertiliser and other organic sources like animal manure (Kechaou *et al.*, 2013; Nguyen *et al.*, 2010).

2.1.5.4 The functions of nutrients

Plant nutrients are quantitatively divided into two categories, macronutrients and micro nutrients.

- **Macronutrients**

Macronutrients are further classified as either primary or secondary nutrients. Primary nutrients include phosphorus, nitrogen and potassium. These three nutrients are required in large quantities. The primary nutrients are nitrogen, phosphorus and potassium. Secondary nutrients are magnesium, sulphur and calcium. They are taken up by plants in considerable amounts (Kechaou *et al.*, 2013).

- **Micronutrients or trace elements**

The micronutrients or trace elements include iron, boron, molybdenum, zinc, manganese, chlorine and copper (Nguyen *et al.*, 2010). These can be exogenously supplied in minute quantities if they are not sufficient in the soil solution. (Kechaou *et al.*, 2013).

- **Harmful elements**

Application of some microelements at higher levels than recommended can be toxic for plants. In its regulations regarding fertilisers, the Department of Agriculture, Forestry and Fisheries in South Africa (Department of Agriculture 2012) gazetted the maximum levels of potentially harmful elements allowable in fertilisers (Table 2-1).

Table 2-1; Gazetted maximum levels of harmful elements

Elements	Concentration (mg/kg)
Cadmium	20
Chrome	1750
Copper	750
Mercury	10
Nickel	200
Lead	200
Zinc	2750
Arsenic	20
Selenium	15

2.2 TECHNOLOGIES FOR PRODUCTION OF FISH FERTILISERS

Basically two technologies are employed in manufacturing fish fertilisers; fish emulsion and fish protein hydrolysates (Beckley & Barrie, 2010; Milnes, 2015). Although fish meal can also be used as a fertiliser, it is considered an expensive product for the purpose (Abowei & Tawari, 2011).

2.2.1 Production of fish emulsion

Fish emulsion is produced concurrently with fish meal (Beckley & Barrie, 2010). In the wet process for fish meal manufacturing (refer to section 2.1.1), the sticky water produced is concentrated by evaporation to 30-40% solids (Abowei & Tawari, 2011; Koning, 2005; Pigott, 1967). This concentrate is the emulsion that can be used as fertiliser after being stabilised with formic acid (Milnes, 2015).

2.2.2 Production of FPH

Fish protein hydrolysates can be produced either chemically or enzymatically and the properties of the resultant FPH will differ according to the hydrolysis method employed. Chemical hydrolysis employs either a strong acid or alkali at high temperatures, while enzymatic hydrolysis employs a proteolytic enzyme or enzyme mixture (Fountoulakis & Lahm, 1998; Ghaly *et al.*, 2013; Ramakrishnan *et al.*, 2013).

2.2.2.1 Chemical methods for hydrolysis

Acid hydrolysis

Hydrochloric acid (HCl) is commonly used for hydrolysis purposes since it is easy and convenient to apply as a reagent (Fountoulakis & Lahm, 1998; Ghaly *et al.*, 2013). However, other acids have also been used. Beckley and Barrie (Beckley & Barrie, 2010) applied phosphoric acid to hydrolyse fish by-products to manufacture liquid fertiliser, whilst Zhu Xian and colleagues (Xian *et al.*, 2008) utilised hydrochloric acid to produce amino acids from fish proteins hydrolysis in subcritical water.

Acid hydrolysis is affected by the concentration and type of acid, temperature (120-140°C), pressure (220-310 kPa), and time of hydrolysis (2-8 h) (Pasupuleti & Braun, 2010). All of these independently and combined will have an impact on quality of the product. The specific parameters

might differ with the acid and product end use but the basic process remains the same. The raw material is heated at high pressure and temperature for a set time. At the required DH, the temperature and the pressure are reduced to end the hydrolysis.

Acid hydrolysis has mostly been confined to the food and pet industries for manufacturing flavour enhancers because it destroys some essential amino acids like methionine, cysteine and tryptophan. Amino acids like glutamine and aspargine are also converted to glutamic acid and aspartic acid (Bucci & Unlu, 2000). The process also adds significant amounts of salt, which is detrimental to the growth of microorganisms. For this reason some of the manufacturers remove salt partially or completely by precipitation, nanofiltration and/or ion exchange resins (Nagodawithana, 1998; Pasupuleti & Braun, 2010). Another drawback of the acid hydrolysis process is its high operational temperature and pressures. Such conditions would require high capital investment for equipment that can withstand the high temperatures and pressure. Safety standards also have to be high when working with strong acids.

Alkaline hydrolysis

There are limited commercial applications of alkaline protein hydrolysates in biotechnology (Ghaly *et al.*, 2013; Pasupuleti & Braun, 2010). Like acidic hydrolysis, alkaline hydrolysis is also common in the food industry for manufacturing flavour enhancers. The alkaline hydrolysis process starts by heating the protein for solubilisation, followed by the addition of alkaline agents like calcium, sodium or potassium hydroxide and maintaining the temperature to a desired set point (typical range 25–55°C)(Pasupuleti & Braun, 2010). The hydrolysis will be continued for several hours until it reaches the desired degree of hydrolysis and then terminated by reducing the temperature.

Alkaline hydrolysis has several drawbacks that makes it less applicable. According to Kristinsson and Rasco (Kristinsson & Rasco, 2000) there is removal of the hydrogen atom from the alpha carbon atom in alkaline solutions during hydrolysis, which results in some side reactions including the racemisation of L-amino acids into D-amino acids, which do not form proteins (Fountoulakis & Lahm, 1998; Kristinsson & Rasco, 2000). There is also loss of cysteine, serine, and threonine via β-elimination reactions and formations of undesirable products like lysinoalanine, ornithinoalanine, lanthionine, and β-amino alanine due to the splitting of disulphide bonds.

(Kinsella, 1976; Lahl & Braun, 1994; Linder *et al.*, 1995). According to Krause and Schmidt (Krause & Schmidt, 1974), alkaline hydrolysis reaction products are also inhibitory to proteolytic enzymes, thus they limit the hydrolysis reaction rate.

In conclusion, the major drawback of chemical hydrolysis is that it is difficult to control, thereby producing products which are chemically and functionally inconsistent (Fountoulakis & Lahm, 1998; Linder *et al.*, 1995). It also produces nutritionally and functionally poor products, making the products useful only as flavour enhancers (Pasupuleti & Braun, 2010).

2.2.2.2 Enzymatic hydrolysis methods

In enzymatic hydrolysis, enzymes are utilised to hydrolyse peptide bonds. Enzymatic hydrolysis methods are a preferable alternative since they do not destroy any constituent amino acid in the fish, and occur at moderate conditions of pH and temperature (Fountoulakis & Lahm, 1998; Ghaly *et al.*, 2013). The process can also remain wholly organic, which is a major advantage when considering environmental sustainability. Enzymatic hydrolysis can proceed either by application of endogenous enzymes present in the guts and muscle of the fish or exogenous enzymes acquired from other sources (Kristinsson & Rasco, 2000). A description of each of these process routes is given below.

Production of autolysates

An autolytic process is a simple operation that employs the fish's digestive enzymes for hydrolysis (Shahidi *et al.*, 1995). The digestive enzymes involved include the major proteases of the digestive tract and fish viscera. Autolysis generally has an end product of a viscous liquid, comprising mostly free amino acids and peptides of low molecular weight (Kristinsson & Rasco, 2000). Two process routes are reported in literature and will be described; acidic autolysis and fermentation autolysis (Abowei & Tawari, 2011; Ghaly *et al.*, 2013).

Acidic autolysis

In this method, fish by-products are hydrolysed in an acidic environment. Strong inorganic acids (e.g. hydrochloric, sulphuric and phosphoric acid) or organic acids like formic acid and acetic acid are usually mixed with the substrate, creating a low pH environment which eliminates putrefying

and pathogenic micro-organisms (Arruda *et al.*, 2007; Ghaly *et al.*, 2013; Kristinsson & Rasco, 2000). The choice of acid depends on a variety of factors. Organic acids, although generally more expensive than the mineral acids, possess antimicrobial properties that enable them to stabilise the silage at a higher pH of between 3.5 and 4, whereas mineral acids are effective at pH 2 (Raa & Gildberg, 1982). Sometimes a mixture of acids is used to harness the benefits of utilising a cheaper mineral acid, as well as an antimicrobial organic one (Abowei & Tawari, 2011; Kompiang, 1981; Lo *et al.*, 1992). At pH 4, the serine proteases are generally inactive, but pepsin and the catheptic enzymes are highly active (Kristinsson & Rasco, 2000).

Fermentation (biological silage)

In fermentation autolysis fish processing by-products are chopped, minced and mixed with a fermentable sugar and a bacteria starter culture (Kristinsson & Rasco, 2000). An example of such a sugar is 5% (w/w) sugar beet molasses. In most cases the lactic acid bacteria (LAB) are added to ferment the added carbohydrate source to produce lactic acid and antimicrobial substances which together provide preservation against competing pathogenic microflora (Abowei & Tawari, 2011; Raa & Gildberg, 1982). The fermentation autolysis produces a mixture of lipids, solubilised proteins, peptides, amino acids and water soluble components (Dong *et al.*, 1993).

Controlled enzymatic hydrolysis with exogenous enzymes

Exogenous enzymes are added to speed up the hydrolysis process and make it controllable, and therefore reproducible (Kechaou *et al.*, 2013; Kim & Lee, 1987). There are a wide variety of proteolytic enzymes commercially available from animal, plant and microbial origins. The most commonly used enzymes for protein hydrolysates from animal sources are pancreatin, trypsin, and pepsin whilst from plant sources are papain and bromelain, and a wide range of proteases are available from bacterial and fungal sources (Benjakul & Morrissey, 1997; Ghaly *et al.*, 2013; Guerard *et al.*, 2001; Shahidi *et al.*, 1995). The choice of enzyme depends on the protein source and end user requirements (Bhaskar *et al.*, 2008; Kristinsson & Rasco, 2000). For example, if the protein has a higher content of hydrophobic amino acids then the enzyme of choice should be the one that preferentially cleaves the hydrophobic amino acids (Pasupuleti & Braun, 2010). Some commercially available proteases are shown on table 2.2. As can be seen on the table, the vegetable enzymes like papain from papaya, and the fungal (SEBDigest F59P) are advantageous in that they

are optimally active at near neutral pH and at 70°C. A temperature of 70°C is well above the survival temperature of spoilage bacteria (Mackie, 1981). The FPH produced will also not need to be neutralised since the pH is near neutral. Neutralisation introduces salts in the final product.

Table 2-2; Optimal conditions for some commercially available enzymes (Mackie, 1981)

Enzyme	pH	Temperature	Enzyme category
Pepsin	2.0	55	Endopeptidase
SEBPro XL (Papain)	6.5	70	Exopeptidase
Alcalase	8.5	55	Endopeptidase
Neutrase	6.0	50	Endopeptidase
SEBDigest F59P (Fungal)	7.0	70	Endopeptidase

Enzyme specificity, operating conditions and the attained DH will determine the variety of peptides that are generated (Kechaou *et al.*, 2013), on which the properties of the resultant hydrolysate will in turn depend (Kim & Mendis, 2005). The hydrolysate characteristics determine its usability as a fertiliser, in animal feeds, human foods or nutraceuticals (Benjakul & Morrissey, 1997; Ghaly *et al.*, 2013; Kim & Mendis, 2005).

Comparing the technologies for production of fish fertiliser

Fish emulsion, from the wet process is primarily composed of water soluble nutrients and contains a relatively less concentration of oil soluble nutrients than FPH fertiliser (Beckley & Barrie, 2010; Milnes, 2015). It is also characterised by an offensive odour from decomposing proteins since it is exposed to very high temperatures (95°C) during processing.

FPH fertilisers also have a wide range of differences setting them apart. When compared to fish silage, controlled hydrolysis possesses an advantage of controllability and reproducibility (Pasupuleti & Braun, 2010). With a good background knowledge, exogenous enzymes can be selected to give a pre-determined result (Benjakul & Morrissey, 1997; Guerard *et al.*, 2001), where an enzyme quantity can be predicted to achieve a certain DH within a specific time. The hydrolysis time, however will determine the amount of resultant bond cleavage.

Silage production and added exogenous enzymatic hydrolysis also differ notably on the production economics. Since autolysis rely on internal enzymes, it does not incur enzyme costs (Kristinsson & Rasco, 2000; Raa & Gildberg, 1982). According to Arruda and colleagues (Arruda *et al.*, 2007) the autolysis technology is relatively simple and the production cost is nearly independent of the scale of production. This makes silage production a suitable production route in cases where relatively low amounts of fish processing waste does not justify high capital outlay of alternative processing plants, or where processing waste is only available seasonally or produced in remote areas (Dong *et al.*, 1993). The controlled hydrolysis with added exogenous enzymes requires a relatively higher degree of process control, making the technology more sophisticated than silage production (Shahidi *et al.*, 1995) and therefore incur higher operating and capital expenditure costs.

Although controlled hydrolysis with added exogenous enzymes is more costly than autolysis, it is more preferred due to its controllability and reproducibility. This brings consistence to industrial applications. The process can also be optimised for parameters like temperature, pH and enzyme concentration, to bring down the costs and maximise on process profitability.

2.3 HYDROLYSIS BY EXOGENOUS ENZYMES

2.3.1 A brief introduction to enzymes

Enzymes are biological polymers that catalyse biochemical reactions (Bugg, 2004). The vast majority of enzymes are proteins, however there are some catalytic ribonucleic acid (RNA) molecules called ribozymes (Bugg, 2004; Robinson, 2015). Enzymes are effective and highly specific catalysts (Robinson, 2015), catalysing the conversion of one or more compounds called substrates into one or more compounds called products. Like all catalysts, enzymes are neither consumed nor altered during catalysis.

2.3.2 Classification of Enzymes:

Enzymes are divided into six major classes (Bisswanger, 2002; Bugg, 2004; Robinson, 2015):

- ***Oxidoreductases***: involved in oxidation and reduction reactions, e.g. oxidases, dehydrogenases, oxygenases, peroxidases.
- ***Transferases***: transfer functional groups, e.g. amino or phosphate groups, e.g. aminotransferases,
- ***Hydrolases***: Catalyse hydrolysis of the substrate, e.g. lipase, maltase, protease. Proteases hydrolyse proteins, cleaving peptide bonds and resulting in the production of amino acids and/or peptides as products.
- ***Lyases***: add or remove elements of water, ammonia, or carbon dioxide to form double bonds, e.g. decarboxylases.
- ***Isomerases***: catalyse the rearrangements of atoms within a molecule to give its isomer, e.g. glucose to fructose
- ***Ligases***: join two molecules, e.g. carboxylases and synthetases.

2.3.3 Proteolytic Enzymes

A proteolytic enzyme is an enzyme that conducts proteolysis, which is protein catabolism by cleaving the peptide bonds linking amino acids in a protein (Gupta & Khare, 2007). Proteolytic enzymes are also referred to as peptidases, proteases or proteinases. They hydrolyse the chain of amino acids, usually only at certain points depending upon the specificity or nature of the enzyme (Bugg, 2004; Devi *et al.*, 2008). Prior to the hydrolysis, the enzyme molecule becomes associated

with, and binds to the protein and at a later stage the protein chain is broken at the peptide bonds to release fragments of peptides and single amino acids (Bisswanger, 2002; Mackie, 1981).

Proteolytic enzymes belong to the hydrolase class of enzymes and are grouped into the subclass of the peptide hydrolases or peptidases. Depending on the site of enzyme action the proteases can also be subdivided into exopeptidases or endopeptidases (Mótyán *et al.*, 2013).

2.3.3.1 Exopeptidases

Exopeptidases catalyse the hydrolysis of the peptide bonds near the *N*- or *C*-terminal ends of the substrate (Mótyán *et al.*, 2013). Aminopeptidases can liberate single amino acids, dipeptides (dipeptidyl peptidases), or tripeptides (tripeptidyl peptidases) from the N-terminal end of their substrates. Single amino acids can be released from dipeptide substrates by dipeptidases or from polypeptides by carboxypeptidases while peptidyl dipeptidases liberate dipeptides from the C-terminal end of a polypeptide chain (Sawant & Nagendran, 2014).

2.3.3.2 Endopeptidases

Endopeptidases hydrolyse peptide bonds of non-terminal amino acids. The endopeptidases are further classified into four major classes; serine, cysteine, aspartic proteases and metallo-proteases depending on the active site functional group (Gupta & Khare, 2007; Vishalakshi & Dayanand, 2009). The proteases are be further categorised as acidic proteases, neutral proteases, or alkaline proteases depending on their pH optima

2.3.4 Process description

Enzymatic proteolysis and solubilisation of proteins has been extensively researched and described by different workers (Benjakul & Morrissey, 1997; Benjakul *et al.*, 2007; Bhaskar *et al.*, 2008; Kechaou *et al.*, 2013; Ovissipour *et al.*, 2010). Although the procedures vary slightly from one researcher to the other, the framework remains the same. The basic principle remains that the fish by-products are exposed to proteases at conditions favourable to hydrolysis (pH and temperature), and allowed to hydrolyse for some set time, before the reaction is terminated by either heating or pH adjustment.

Some researchers cooked the fish by-products to deactivate endogenous enzymes (Iizuka & Konishi, 1995; Ovissipour *et al.*, 2009; Ovissipour *et al.*, 2010) whereas others prefer to leave

them active in the substrate. Cooking the substrate to inactivate endogenous enzymes is important, mostly to gain total control of the hydrolysis process by excluding endogenous enzyme action, as well as for comparative purposes of the added exogenous proteases. However, the contribution of endogenous enzymes could be critical in commercial set ups if they also take part in substrate degradation. According to Slizyte and colleagues (Slizyte *et al.*, 2005), endogenous enzymes inactivation causes formation of protein–lipid complexes which lead to increased amount of sludge, reduced FPH yield and higher amount of lipids in FPH.

Iizuka and Konish (Iizuka & Konishi, 1995) removed oils from the reaction mixture before hydrolysis arguing that the oils prevent fertilising components from absorbing onto the plant. However, other authors like Milnes and colleagues (Milnes, 2015) and Beckley (Beckley & Barrie, 2010) are proponents of the inclusion of oils in the final hydrolysate. By removing the oils, they argue that some oil soluble nutrients are removed, thereby reducing the nutritional value of the hydrolysate.

However, oils are extremely susceptible to oxidation (Mackie, 1981), therefore for preservation purposes, it is important that they be removed from the hydrolysates. If they cannot be removed, then they will need to be stabilised with anti-oxidants (Gildberg, 1993). Examples of such antioxidants are butylated hydroxytoluene, butylated hydroxyanisol (Hoyle & Merritt, 1994) and propylgallate. Oxidation of lipids as a result of reactions with basic groups in proteins may darken the fish protein hydrolysates (Kristinsson & Rasco, 2000). A generalised block diagram for enzymatic hydrolysis is shown on Figure 2-1.

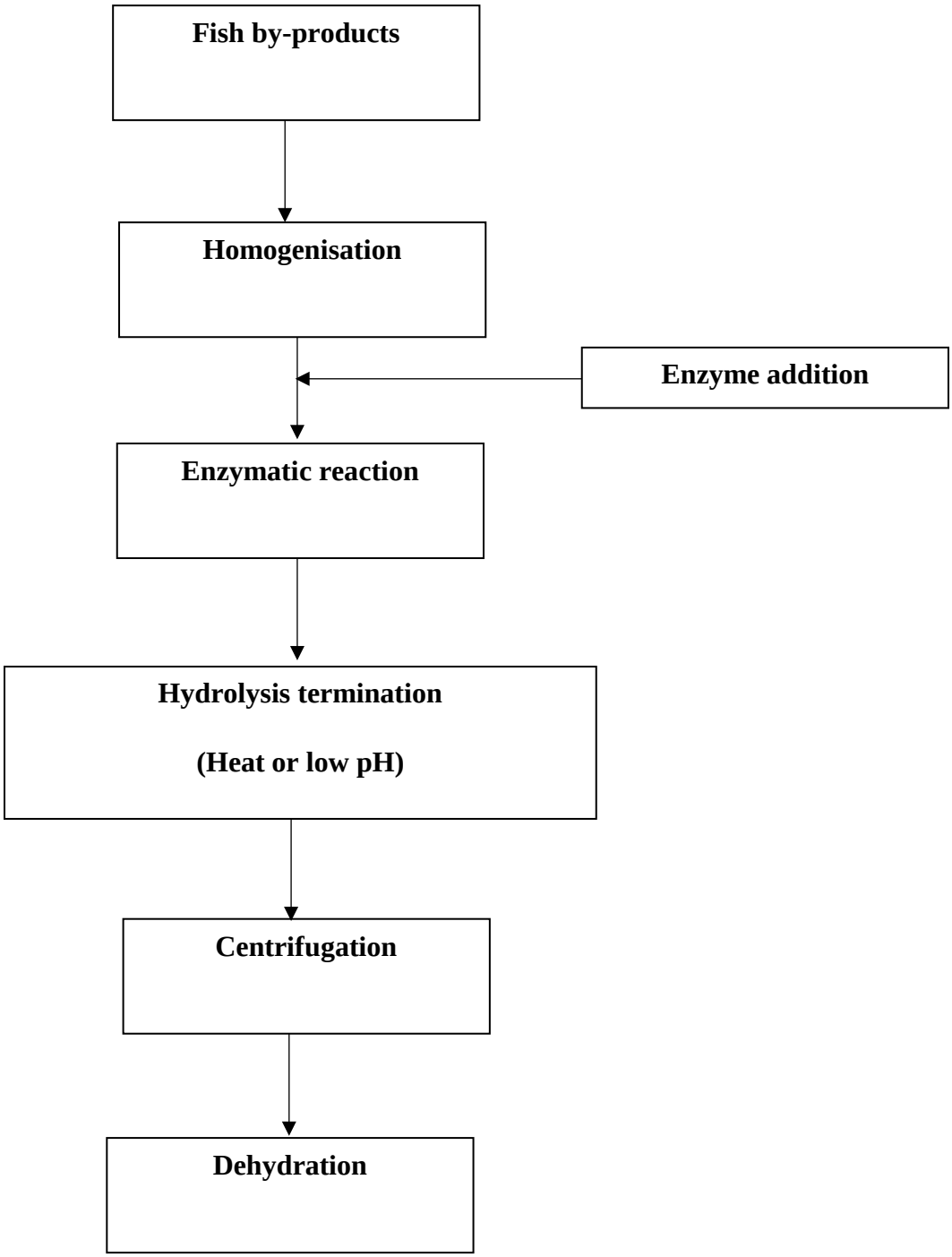

Figure 2-1; General block diagram for enzymatic hydrolysis (Kristinsson & Rasco, 2000)

2.3.4.1 Mincing and water addition

This is a preparatory stage for the hydrolysis. It exposes the substrate for enzyme action. The fish processing by-products are ground and mixed with water in equal ratios (Guerard *et al.*, 2001; Kechaou *et al.*, 2013) or any other ratio as per design and homogenised (Kristinsson & Rasco, 2000). Addition of water, although it facilitates good mixing and enzyme access to substrate, introduces more water into the reaction mixture. The water will have to be removed during dehydration to reduce the bulkiness of the product, thus increasing energy consumption and making the process more costly.

Sometimes the minced material is mixed with a buffer solution for pH control. Such buffers as phosphate buffer (Ovissipour *et al.*, 2009; Ovissipour *et al.*, 2010; Rebeca *et al.*, 1991) and boric acid-NaOH buffer (Baek & Cadwallader, 1995) are used. However according to Kristinsson (Kristinsson & Rasco, 2000), the buffer salts may negatively affect the foaming and emulsifying properties of the hydrolysates produced.

2.3.4.2 Conditioning

The mixture is secured in a reaction vessel in a water bath and temperature is adjusted to set values. The set up usually carries an overhead stirrer, a thermometer for temperature control, a pH electrode and an auto titrating device for pH regulation, if the system is not buffered (Kristinsson & Rasco, 2000). At the required temperature, the pH of the hydrolysis mixture is adjusted to the desired value by adding a base or an acid. Processing conditions of temperature and pH are normally optimised according to the characteristics of the enzyme and substrate mixture (Pasupuleti & Braun, 2010).

2.3.4.3 Hydrolysis

At the correct pH and temperature, an exogenous enzyme is added in predetermined concentrations depending on the desired rate of hydrolysis (Benjakul & Morrissey, 1997; Guerard *et al.*, 2001; Kechaou *et al.*, 2013; Opheim *et al.*, 2015). For any particular enzyme-substrate system, the hydrolysis process is influenced by at least five factors (protein substrate concentration, enzyme-substrate ratio, pH, temperature and time) (Adler-Nissen, 1976). The enzyme will digest the substrate into amino acids and simpler peptides. End of hydrolysis is signified either by time or degree of hydrolysis. When the reaction has reached its completion, it is then terminated.

2.3.4.4 Tracking the hydrolysis reaction

To monitor the hydrolysis process, the DH is employed. The DH is a percentage measure of the amount of cleaved peptide bonds (Nielsen *et al.*, 2001). Measuring the DH for process monitoring is advantageous since it reliably determines the properties of a protein hydrolysate in any given protein-enzyme mixture (Kristinsson & Rasco, 2000).

Measuring the DH

DH is defined as the percentage of cleaved peptide bonds:

$$DH = \frac{number\ of\ peptide\ bonds\ cleaved}{total\ number\ of\ peptide\ bonds} X100\% \qquad \textit{Equation 1}$$

The total number of peptide bonds depends on the substrate amino acid composition (Kristinsson & Rasco, 2000; Nielsen *et al.*, 2001). Different methods are reported in literature for DH measuring. The common ones include the pH-stat (Jacobsen *et al.*, 1957), osmometry (Adler-Nissen, 1984), soluble nitrogen content (Margot *et al.*, 1994), the trinitro-benzene-sulfonic acid (TNBS) (Adler-Nissen, 1979) and the o-phthaldialdehyde (OPA) (Nielsen *et al.*, 2001) methods.

The pH-stat method

The pH-stat method as described by Jacobsen and colleagues (Jacobsen *et al.*, 1957) measures the DH by calculating the volume of the base or acid consumed by the reaction in maintaining the pH constant. The molarity of either base or acid used is also taken into consideration. The DH is calculated by the following equation (Equation 2);

$$DH = \frac{B.NB}{\alpha.h_{tot}.MP} * 100. \qquad \textit{Equation 2}$$

Where; B is the base or consumption in ml, NB is the normality of the base or acid, α is the average degree of dissociation of the –NH groups or COOH groups and MP is the mass of protein in grams (%N × 6.25).

To calculate the degree of dissociation, equation 3 is used;

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \qquad \textit{Equation 3}$$

According to Kristinsson and Rasco (Kristinsson & Rasco, 2000), equation 3 is only valid at neutral and alkaline pH. It is under such conditions that hydrolysis reactions release protons (H^+) and, as pH control measure, consume proportional amounts of base to the number of split peptide bonds (Adler-Nissen, 1977). This limits the pH-stat method to hydrolysis conditions with a pH of 7 and above (Adler-Nissen, 1986; Nielsen *et al.*, 2001), making it irrelevant to acidic environments, which is its major drawback since some hydrolysis reactions are carried out at acidic pH.

The Trichloroacetic acid (TCA) method

The TCA method, as reported by Margot and colleagues (Margot *et al.*, 1994) is used to monitor the hydrolysis process by measuring the concentration of dissolved nitrogen in trichloroacetic acid (TCA). The TCA method approximates the DH of protein hydrolysates by comparing soluble nitrogen in the aqueous 10% TCA solution to the total amount of protein in sample. Hydrolysates samples are mixed with 20% TCA to create two 10% TCA fractions, one soluble and another insoluble. By centrifuging the mixtures and analysing the supernatant for nitrogen, the DH is then calculated using the following equation (Equation 4):

$$DH = \left(\frac{10\%\ TCA\text{–}soluble\ N\ in\ sample}{Total\ N\ in\ sample}\right) X\ 100 \qquad \textit{Equation 4}$$

The major drawback to the TCA method is that it functions well mostly with the use of endo-peptidases, since the cleavage of the same amount of bonds by an endoprotease system and an exoprotease system does not result in the same amount of soluble nitrogen (Nielsen *et al.*, 2001).

The TNBS method

The TNBS method measures the total primary amino groups liberated during hydrolysis. The released groups are made to react with the trinitro-benzene-sulfonic acid (TNBS) reagent (Adler-Nissen, 1979). The chromophore resulting from the reaction is then spectrophotometrically measured at 420 nm and the DH is then calculated as reported by Baek and Cadwallader (Baek & Cadwallader, 1995) when investigating crayfish hydrolysis and Benjakul and Morrissey (Benjakul & Morrissey, 1997) when investigating pacific whiting hydrolysis:

$$DH = \left(\frac{L_t - L_o}{L_{max} - L_o}\right) * 100 \qquad \textit{Equation 5}$$

Where; L_t represents the amount of a specific amino acid released after a specific hydrolysis time t, L_0 is the amount of the specific amino acid in the raw material substrate and L_{max} is the maximum amount of the specific amino acid obtained after complete hydrolysis.

However, the TNBS method presents challenges of being a laborious and lengthy procedure, making it difficult to follow the process closely. The TNBS reagent is also unstable and toxic, with a potential to explode if not carefully handled (Nielsen *et al.*, 2001).

The modified OPA Method

The modified o-phthaldialdehyde (OPA) method as reported by Nielsen (Nielsen *et al.*, 2001) is another way of measuring the DH. It is a modification of the OPA method discussed by Church and others (Church *et al.*, 1983), by replacing β-mercaptoethanol with dithiothreitol (DTT), which is more environmentally friendly. The OPA method uses a reaction between amino groups and OPA in the presence of DTT, to form a coloured compound which is spectrophotometrically detectable at 340 nm. Serine is used as a standard in the spectrophotometric analysis.

The modified OPA method was reported to be more accurate and stable than the original OPA method. It is also much faster than the TBNS method and therefore can be used to monitor the DH during hydrolysis, with results being made available two minutes after sampling (Nielsen *et al.*, 2001).

The modified OPA method also overcomes the drawbacks of the osmometry and the TCA method in that it can measure the DH of viscous solutions and any protease type (endo or exo proteases) (Kristinsson & Rasco, 2000). It can also be used to monitor the hydrolysis under acidic or alkaline pH (Nielsen *et al.*, 2001). These qualities of the OPA method made it the method of choice in this investigation.

2.3.4.5 Termination of the hydrolysis reaction

Termination is achieved by inactivating the proteases by heating to a higher temperature (95^{o}C for fifteen minutes) (Guerard *et al.*, 2001; Ovissipour *et al.*, 2010) or by pH adjustment to a value outside the enzyme operating range. The hydrolysate mixture is then left to cool and centrifuged to collect the aqueous phase. The clarified liquid can be used as a fertiliser as is or it may be further treated by drying it into a solid product (Beckley & Barrie, 2010; Iizuka & Konishi, 1995).

2.3.5 Factors affecting enzymatic hydrolysis

Various environmental factors are able to affect the rate of enzyme-catalysed reactions through reversible or irreversible changes in the protein structure (Robinson, 2015). Temperature, pH and enzyme concentration, mass transfer and substrate concentration are some of the critical factors that affect enzymatic hydrolysis.

2.3.5.1 pH

Most enzymes have a characteristic optimum pH at which the rate of the catalysed reaction is maximal, and above and below which the rate declines (Marangoni, 2003; Robinson, 2015). The pH profile is influenced by many factors. As the pH changes, the ionisation of groups both at the enzyme's active site and on the substrate can alter, influencing the rate of binding of the substrate to the active site. These effects are often reversible, however, at extreme pH, the bonds which maintain the tertiary structure, including the active site, are disrupted and the enzyme is irreversibly denatured (Bisswanger, 2002; Bugg, 2004).

2.3.5.2 Temperature

Enzymes work rapidly at their optimum temperatures (Bisswanger, 2002; Robinson, 2015). The effects of temperature on enzyme activity involve two opposite forces acting simultaneously. When the reaction temperature is increased, the rate of molecular movement increases, thereby increasing the rate of reaction. However, there is a simultaneous progressive inactivation resulting

from the denaturation of the enzyme, which becomes more pronounced with increasing temperature, culminating in an apparent temperature optimum (T_{opt}) (Bisswanger, 2002; Bugg, 2004). Increasing the temperature above this optimum results in falling of the rate of reaction due to denaturation. Since thermal denaturation is time dependent, the enzyme's optimum temperature also depends on time of reaction. Denaturation is the breakdown of hydrogen bonds and sulphide bridges which maintain the shape of the enzyme active site. As a result, no more enzyme-substrate complexes will be able to form, rendering the enzyme ineffective (Marangoni, 2003).

The temperature at which denaturation becomes important varies from one enzyme to another. Normally it is negligible below 30°C, and starts to become appreciable above 40°C (Robinson, 2015). Typically, enzymes derived from microbial sources show much higher thermal stability than those from mammalian sources. Enzymes derived from extremely thermophilic microorganisms, such as thermolysin (a protease from *Bacillus thermoproteolyticus*) and Taq polymerase (a DNA polymerase from *Thermus aquaticus*), might be completely thermostable at 70°C and still retain substantial levels of activity even at 100°C (Bisswanger, 2002; Bugg, 2004).

The Temperature Coefficient, Q_{10}

The temperature coefficient of an enzyme describes the effect of temperature on the rate of a chemical reaction (Marangoni, 2003). It is a factor by which a reaction rate will increase when an increase in temperature of 10°C is effected.

$$Q_{10} = \frac{(Rate\ of\ reaction\ at\ (T+10)^oC)}{Rate\ of\ reaction\ at\ T^oC} \qquad Equation\ 6$$

Most enzymes have a Q_{10} of between 2 and 3 (Byrne, 1999), which is a doubling or a trebling effect if the temperature is not so high that it causes denaturation.

2.3.5.3 Enzyme concentration

Enzyme-substrate complexes are formed at the active sites of the hydrolysing enzyme (Bugg, 2004; Wilkinson, 1971). At low enzyme concentrations, the active sites are the limiting factor of the reaction and the substrate is said to be in excess. The reaction is also referred to as a zero order reaction since it is independent of substrate concentration. Increasing the enzyme concentration

therefore increases the rate of hydrolysis until a point where the available active sites are equal to the substrate. Increasing the enzyme concentration beyond this point will not have further effect on the rate of hydrolysis since the substrate concentration will have become the limiting factor (Marangoni, 2003).

2.3.5.4 Substrate concentration

Initially, increasing the substrate concentration considerably increases the rate of reaction. However, these effects of substrate concentration on the reaction rate start to decline with a continued increase in substrate concentration until it becomes of little or no further effect (Marangoni, 2003). The enzyme is then said to have reached its substrate saturation point, and therefore its maximum rate of reaction, referred to as the maximal velocity (V_{max}). The Michaelis-Menten equation (equation 7) describes the relationship between V_{max} and substrate concentration, as well as the Michaelis constant (K_m).

$$V_o = \frac{V_{max}[S]}{[S]+K_m} \qquad \textit{Equation 7}$$

Where V_o is the initial rate of reaction and [S] is the substrate concentration. K_m, the Michaelis constant is defined as a measure of the enzyme's affinity to its substrate. An enzyme with a low K_m value has a high substrate affinity and requires a small amount of substrate to be saturated, hence maximum velocity occurs at relatively low substrate concentrations. For an enzyme with a high K_m value, the maximum velocity is achieved with a high substrate concentration. Such an enzyme is said to be of low substrate affinity (Wilkinson, 1971).

2.3.5.5 Time of reaction

Reaction time is also an important factor in enzymatic hydrolysis. A longer hydrolysis time allows the enzyme to act more extensively, therefore increasing the extent of hydrolysis (Haslaniza *et al*., 2010). Other authors like Bhaskar (Bhaskar *et al*., 2008) and Ovissipour and colleagues (Ovissipour *et al*., 2009) also investigated and reported similar findings where the degree of hydrolysis increased with the increase in incubation time.

2.3.5.6 Mixing

The agitation rate also needs to be controlled for effective mixing. Purwanto and colleagues (Purwanto *et al.*, 2009) reported that low and excessively high agitation speeds negatively affect the productivity of enzymatic hydrolysis process because enzymes are sensitive to mechanical shear stress. Similar results were also reported by Elibol and Ozer (2000) that at low agitation speeds, mass transfer between enzymes and the substrate is insufficient, resulting in low hydrolysis rates. However at excessively high agitation rates, the reaction rate is also depressed since high mechanical forces perturb the enzyme protein complex, leading to enzyme inactivation (Elibol & Ozer, 2000). Medium agitation rates (e.g 200 rpm) are sufficient for hydrolysis (Ovissipour *et al.*, 2010; Nilsang *et al.*, 2004).

2.4 CONCLUSION FROM THE LITERATURE

The following conclusions can be made from the literature review;

Enzymatic hydrolysis has been used for recovery of fish proteins in fish processing by-products for many years, producing fish protein hydrolysates (FPH) which have many applications. Enzymatic hydrolysis generates a mixture of free amino acids and peptides of varying sizes with improved solubility, functionality and bioavailability. The major desirable functional properties of hydrolysates include solubility, foaming, water holding, and emulsifying, as well as sensory properties. These are a function of the type of substrate, enzymes employed and the DH attained by the hydrolysis. It is therefore important to select the right protease for a specific substrate to achieve the intended results. The DH, also being a critical determinant of the FPH characteristics, has to be monitored throughout the hydrolysis process.

Enzymatically produced FPH has been used as a crop fertiliser for enhanced yields and productivities. The critical parameters when FPH is used as a fertiliser are the free amino acids (FAA) and the DH. The free amino acids resultant from enzymatic hydrolysis have been reported to be directly absorbed by plants through their roots and leaves to function as building blocks for plant proteins. This makes the FPH a potential folia fertiliser, as well as a soil additive. Thus it is important that the FAA in FPH be defined as a quality control parameter.

The DH is affected by the reaction temperature, pH and concentration of the protease employed. Each combination of substrate and protease have optimum values for temperature and pH at which highest rates of hydrolysis are achieved. Manipulating these reaction parameters and maintaining them at the optimised conditions during enzymatic hydrolysis has been proven to be important. Temperatures and pH outside the proteases' optimum range denatures the proteases, and therefore hinder the hydrolysis reactions. These optimum conditions have to be investigated for each substrate/protease combination as they vary from one hydrolysis combination to the other.

Predetermination of the DH by manipulating the hydrolysis conditions and enzyme concentration has been reported. This could make the commercial hydrolysis process easier to monitor since the need to measure the DH oftenly will be minimised. A known enzyme concentration will be added to hydrolyse the substrate for a specific time and give a predetermined DH. There is need for an

investigation to ascertain these relationships between the enzyme, pH, and temperature over a specific time period.

Proteases have been reported to work both individually and in combinations. Due to a wide variety of proteases available on the market, there exists no record of complementary proteases, therefore there is need for an investigation on the possible combinations of the selected proteases. This work will investigate possible synergistic relationships between enzymes.

From the literature survey, it was gathered that the best way to present this study is to divide it into three phases, phase 1, 2 and 3. The first phase's main objective was to determine the optimum operating conditions of the two selected enzymes, SEBPro XL and SEBDigest F59P on rainbow trout heads as substrate. Three factors, pH, temperature and enzyme concentration, which influence the hydrolysis process were investigated as the independent variables, with the DH as the dependent variable.

The second phase was process development using different enzyme combinations at the determined optimum pH and temperature of the two enzymes in different enzyme combinations to increase DH and establish potential synergistic relationship between the combinations. Combination treatments and the two individual enzymes were designed and applied for hydrolysis. Their resultant FPH were analysed and compared for mostly DH and FAA to determine the best performing enzyme treatment, which was then used in the next phase (phase 3) of the investigation.

The third phase of this work served to increase the DH to at least 45%. In this phase, the selected enzyme treatment from phase 2 was applied at varying enzyme concentrations to determine the concentration that will achieve at least 45% DH within 4 hours of hydrolysis. Relationships were also determined between the E/S, time and DH.

3. PHASE 1: FACTOR OPTIMISATION

3.1 INTRODUCTION

The main aim of this phase was to determine the optimum operating conditions of the two selected enzymes, SEBPro XL (a papain enzyme) and SEBDigest F59P (a fungal enzyme) on rainbow trout heads as substrate. Three factors, pH, temperature and enzyme concentration were investigated as the independent variables, whilst the DH was the dependent variable. A response surface methodology, central composite design was employed to optimise the three factors.

3.2 MATERIALS AND METHODS

3.2.1 Feed preparation

Rainbow trout (*Oncorhynchus mykiss*) heads used as substrate were supplied by Molapong Aquaculture in the Winelands region, Cape Town, South Africa. The material was received frozen and allowed to thaw before being ground in a bowl cutter (model EMS Saarbrucken) at medium speed until homogeneity was achieved. The material was then packaged into 100g packets before being frozen again until use. Samples for proximate analysis were sent to Agricultural Research Council (ARC) - Irene Analytical Services to measure moisture, ash, fat, dry matter, nitrogen and protein content and the results are as shown on table 3-1.

3.2.2 Moisture

Moisture content of the substrate fish by-products was determined using the Association of Official Analytical Chemists (AOAC) official method 934.01 (AOAC, 2000a) whereby a sample containing 2g material was dried to constant weight at 95-100^{o}C under pressure. The loss in weight was reported as moisture.

Table 3-1; Proximate analysis of the raw material

Component	Percentage
Moisture	67.4
Ash	2.2
Dry matter	32.6
Fat	19.7
Nitrogen	1.6
Protein	9.9

3.2.3 Total Protein

The total crude protein in raw material was determined using the Kjeldahl method which measures total organic nitrogen. The organic matter was digested with hot concentrated sulphuric acid, converting all the constituent nitrogen to ammonia. The ammonia was then measured by titration and the total protein was calculated by multiplying the total nitrogen by 6.25 (AOAC, 2000c).

3.2.4 Lipids

Crude lipid content in samples was determined by ether extraction using the Soxhlet method of the AOAC, Official Method 920.39 (AOAC, 2000c).

3.2.5 Ash content

Ash content was estimated by charring a pre-dried sample in a crucible at 600^{o}C until a white ash was formed using the AOAC Official Method 942.05 (AOAC, 2000b).

3.2.6 Preparation of fish protein hydrolysates

3.2.6.1 Chemicals

Potassium hydroxide (KOH) used for pH control was of analytical grade and was supplied by Sigma Aldrich. A 4N solution was made for pH control purposes.

3.2.6.2 Equipment

Equipment utilised in the hydrolysis process consisted of a 34L water bath (model 132 and heated by a model 103 laboratory circulator), supplied by Labotec, South Africa, a variable speed Heidolph overhead stirrer (model RZR 2020), a Heidolph hot plate/magnetic stirrer, a benchtop high speed centrifuge (50 ml tube model H/T16MM), supplied by KIMIX Chemical Company Limited, Hangzhou, China and an automatic pH controller. The automatic pH controller included a Kuntze Neon pH monitor, a Kuntze Zirkon pH probe and a Pulsafeeder PULSAtron Series A Plus electronic metering pump. It was supplied by Dr. Kuntze Instruments GmbH, Germany.

3.2.6.3 Enzymes

Two enzymes were selected for the hydrolysis process, SEBPro XL and SEBDigest F59P. The two enzymes were selected because of their suitability for fish protein hydrolysis (Advanced Enzymes Technologies). The enzymes can also catalyse the hydrolysis at temperatures above 50^{o}C, which is warm enough to suppress significant microbial growth in the reaction mixture (Pasupuleti & Braun, 2010). The two enzymes are also optimally active at near neutral pH (6.5 and 7.5) for SEBPro XL and SEBDigest F59P respectively (Table 2.2). The final product therefore do not need to be neutralised before application, which is advantageous since addition of acids or bases introduces salts into the product, which are detrimental to microorganism growth (Nagodawithana, 1998; Mackie 1981). Data from the suppliers also shows low application rates (0.05% to 0.2% w/w) of substrate concentration (Advanced Enzymes Technologies). Since enzymes are a critical cost driver in enzymatic hydrolysis (Kristinsson & Rasco, 2000), these low application rates make them potential cost-effective catalysts.

SEBPro XL

This enzyme is a food grade protease preparation derived from the sap of an immature papaya fruit, *Carica papaya*. SEBPro XL can operate within a pH range of 6 to 10 and a temperature range of 40 to 80^{o}C. It is a broad pH acting protease which demonstrates exo-protease activity (Advanced Enzymes Technologies). SEBPro XL was received in a 5L container and stored in a refrigerator at below 5^{o}C.

SEBDigest F59P

SEBDigest F59P is a food grade alkaline protease preparation produced by controlled fermentation of selected strain of *Aspergillus oryzae*, a filamentous fungus. It operates optimally within a pH range of 6 to 10 and a temperature range of from 30 to 70°C. SEBDigest F59P demonstrates endo-protease activity (Advanced Enzymes.Technologies), hydrolysing the peptide bonds in protein molecules to liberate polypeptides and peptides of various lengths.

3.2.6.4 Hydrolysis procedure

Hydrolysis experiments were performed in a temperature controlled water bath, using a continuously agitated 500 ml glass beaker as a reaction vessel. The water bath was filled with enough water to immerse the water circulator heating elements and set at predetermined temperatures depending on the experimental run. When the water temperature reached the set point, a 500 ml beaker containing the substrate was then clamped to a beaker holder and kept partly immersed in the water (Figures 3-1 and 3-2). The substrate was prepared by making a 1:1 (w/w) mixture of 100g samples of the fish by-products and 100g of distilled water. The reaction mixture was then stirred for ten minutes to reach the set temperature, after which the pH was adjusted and maintained at a set point by adding 4N KOH through the automatic pH controller. A predetermined amount of enzyme according to the experimental run was added to the reaction mixture whilst the pH and temperature were controlled at set points to begin the hydrolysis process. Constant stirring at 200rpm was maintained throughout the hydrolysis reaction, using an overhead stirrer.

The hydrolysis was allowed to run for 60 minutes and then transferred to a 250ml conical flask which was partly immersed in a 500ml beaker with boiling water on the hot plate (Figure 3-3). The mixture was heated in the 250 ml beaker at temperatures above 95°C for fifteen minutes to inactivate the added enzymes and terminate the enzyme reaction (Nguyen *et al.*, 2010). The hydrolysate mixture obtained was left to cool to room temperature and weighed to determine loss of moisture through evaporation, or gain in volume through KOH addition, which could increase or decrease substrate protein concentration respectively. It was then transferred into 50 ml centrifuge tubes and acidified to a pH less than 3 with phosphoric acid before being centrifuged at 10000rpm for 10 minutes. Acidification was necessary to improve clarity when centrifuging, which was critical for sample spectrophotometric absorbance reading for DH analysis. Turbidity in samples could cause interference when reading absorbances, and therefore affect the accuracy

of results. Centrifugation resulted in four layers, a top oil layer, followed by a sludge layer, then, an aqueous layer, with the bones at the bottom. The oil layer was removed with a plastic disposable pipette and the aqueous layer decanted into sample bottles to be kept for analysis. All the other phases were discarded.

Figure 3-1; Arrangement of equipment in the hydrolysis process set up

Figure 3-2; A pH probe and a base spout secured in the reaction beaker

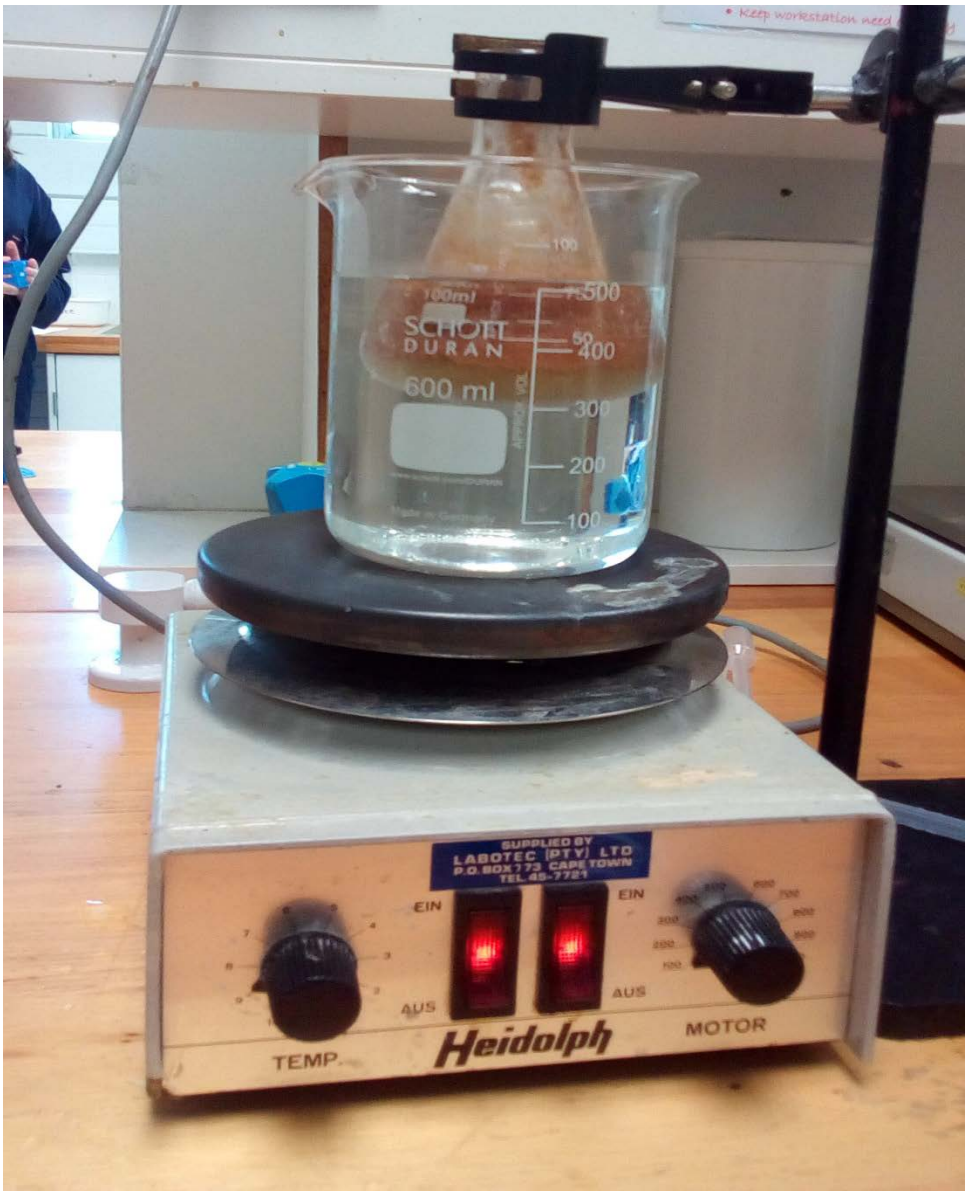

Figure 3-3; Heat inactivation of the enzymes for the hydrolysis process termination

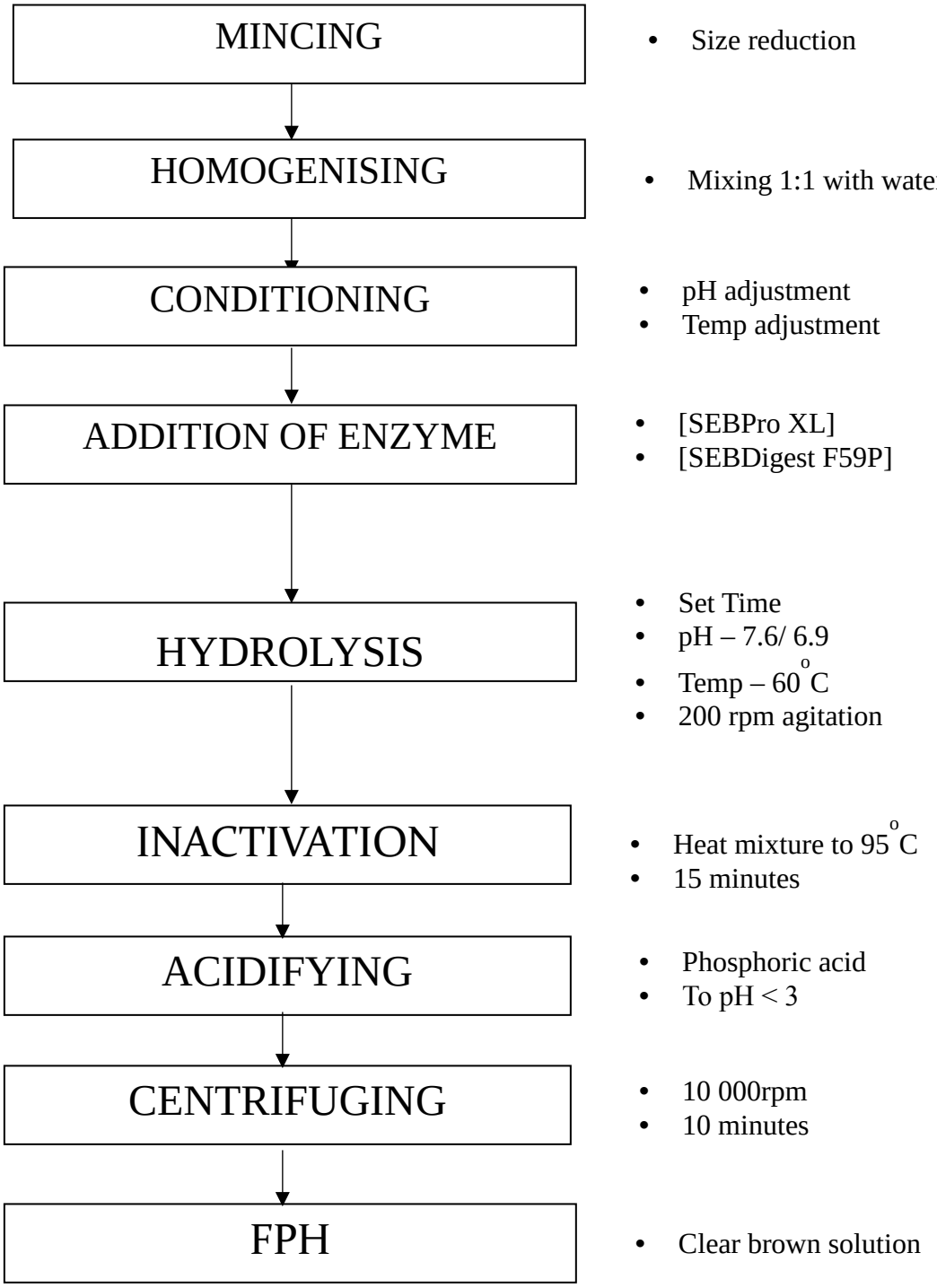

Figure 3-4; Process flow diagram for enzymatic hydrolysis of fish by-products.

3.2.7 Experimental design for optimisation

Response Surface Methodology, (RSM) was used to optimise the hydrolysis conditions. The objective was to simultaneously optimise the levels of process variables to attain the best system performance (Bezerra *et al.*, 2008). Five levels (−α, −1, 0, +1, +α) were applied for each treatment in a circumscribed central composite design (CCD), with replications at the centre. The levels (±α) were star points, (±1) were the high and low levels whilst (0) represented the centre points.

The ranges of independent variables, i.e. enzyme/substrate (E/S) ratio, reaction pH and reaction temperature were based on the recommendations from Advanced Enzyme Technologies Ltd, the enzyme supplier (Figures 9-1 to 9-4) in appendix 9.1.1. The supplier's recommended dosage range for the enzyme SEBDigest F59 was 0.05% to 0.2% w/w and 0.05% to 0.25% for SEBPro XL of the substrate protein concentration. These formed the basis of the experimental design. The CCD applied in experimental design had eight factorial points, six axial points, and six replicates at the centre (Table 9-1) for SEBDigest F59P. The data in Table 9-1 was applied to SEBPro XL but the response surfaces generated showed that the optimum values for the investigated factors were outside the experimental region (Bezerra *et al.*, 2008). The response surfaces however indicated the direction in which the experimental region was supposed to be displaced. A separate CCD for SEBPro XL with eight factorial points, six axial points and four replicates at the centre (Table 9-5) was therefore produced.

The DH (Bhaskar *et al.*, 2008) was the dependent variable to the temperature, pH and E/S, and a model equation was fitted to predict the responses of the hydrolysis system (Bezerra *et al.*, 2008; Ovissipour *et al.*, 2010), at varying levels. The model (equation 8), for DH, the dependent variable (Y) as a function of the three independent variables (X_1, X_2 and X_3) and their interactions was derived using the constant, linear and quadratic regression coefficients of main factors.

$$\boldsymbol{Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j} \qquad \textit{Equation 8}$$

Where;

- Y is the DH, the response variable.
- βo is an intercept term, βii and βij are coefficients predicted by the model.

- X_i and X_j are levels of the independent variables, representing the linear, quadratic, and interaction effects of the X_1, X_2, and X_3 variables on the response (Bhaskar *et al.*, 2008), respectively.

The optimum levels of different independent variables were determined through employing the desirability profile, in which the highest desirability of 1 was assigned to the highest DH observed while a desirability of 0 was assigned to the lowest DH observed. Graphical representations of the profile revealed the optimum DH values.

To check the validity of the model, eight random combinations of the three factors were used to run different experiments. The experiments were run as described in section 3.2.6.4 and the observed DH values from the experimental runs were compared to the model expected values by multiple regression analysis using the Statistica software. Coefficients of determination (R^2) and correlation coefficients (R) resultant from the regression analysis were used to define the relationship between the experimental and validation runs, as well as make conclusion on the fitness of the models to describe the experimental data.

3.2.8 Statistical Analysis

A full factorial response surface methodology, central composite design with 3 factors, 5 levels and 1 block, using the design of experiment (DOE) module of the STATISTICA 13 software package was employed for optimisation of the experimental data. The data were then subjected to a factorial analysis of variance (ANOVA) and tested for significance at 95% confidence level. The linear, quadratic and interaction terms were evaluated by regression analysis and the resultant coefficients were employed to develop response surface models. To evaluate the assumption of normality in ANOVA analysis, the residuals of the data were plotted on normal probability plots.

3.2.8.1 Hypothesis testing

The following hypothesis was tested on the experimental data;

H_o; Temperature, pH, E/S or their interactions will not have a significant effect on the DH

H_1; At least one of the factors or their interactions will have a significant effect on the DH

3.2.9 Optimizing reaction with SEBDigest F59P

The actual and coded values of the factor levels for hydrolysis with SEBDigest F59P are shown on the Table 3-2.

Table 3-2; Coded and actual values for optimisation with SEBDigest F59P

Factor	Levels				
	-α	-1	0	+1	+α
E/S(%w/w), X_1	0.05	0.10	0.15	0.20	0.25
Temperature (^oC) X_2	30	40	55	70	80
pH, X_3	6	7	8	9	10

3.2.10 Optimizing with SEBPro XL

The actual and coded values of the factor levels for hydrolysis with SEBPro XL are shown on the Table 3-3.

Table 3-3; Coded and actual values for optimisation with SEBPro XL

Factor	Levels				
	-α	-1	0	1	+α
E/S(%w/w), X_1	0.2	0.4	0.7	1	1.2
Temperature (^oC), X_2	30	40	55	70	80
pH, X_3	5	6	7	8	9

3.2.11 Determining the Degree of Hydrolysis

The degree of hydrolysis, (Bhaskar *et al.*, 2008), which is the dependent variable, was measured and monitored according to the modified OPA method as reported by P.M. Nielsen and colleagues (Nielsen *et al.*, 2001).

The chemicals used for the OPA method include di-Na-tetra borate decahydrate, sodium dodecyl sulphate (SDS), O-phthaldialdehyde 97%, (OPA), 95% ethanol, dithiothreitol 99% (DTT) and serine. The chemicals were all of analytical grade and were supplied by Sigma-Aldrich, South Africa.

3.2.11.1 Preparing the OPA reagent

7.620g of di-Na-tetraborate decahydrate were weighed into a 250ml conical flask containing 150ml deionised water. 200mg of SDS were then added and the mixture was shaken until the chemicals were completely dissolved. 160mg of OPA were first dissolved in 4ml ethanol (95%) and then transferred to the solution quantitatively by rinsing with deionised water, to which 176mg of DTT were also added and the solution made up to 200ml with deionised water.

The Serine standard

The serine standard was made by diluting 50mg serine in 500ml deionised water (0.9516 meqv/L). By varying the serine concentration from 0.1 to 0.55, a standard curve was produced (Figure 9-11) in appendix 9.3.

Preparation of the hydrolysis sample

0.02g of the hydrolysis sample was diluted in 10ml deionised water. To measure 0.02g of sample solution, an equivalent volume of the hydrolysis sample depending on the specific gravity was measured using a micro-pipette (Acura manual 825, 20-200µl, supplied by Labotec, South Africa). The specific gravity of each sample was determined by withdrawing 5ml of the sample solution using a 5000µl (Pipetman P5000G, supplied by Lasec, South Africa) micro-pipette and weighing the sample using an analytical balance.

3.2.11.2 Procedure

Absorbances were read in a UV/Vis spectrophotometer (model AE-S60 4U) at 340nm.
Deionised water was used as the control.

Standard measuring.

3ml OPA reagent were measured into a 10ml glass test tube and 400µl serine standard were added. The contents of the test tube were shaken for 5 seconds and allowed to stand for exactly 2 minutes before being transferred to a 3ml disposable polystyrene cuvette and the solution's absorbance read at 340 nm in the spectrophotometer.

Blank measuring

3ml OPA reagent were measured to a test tube and 400µl of deionised water were then added and the contents were shaken for 5 seconds. After standing for exactly 2 minutes, the solution was transferred to a cuvette and its absorbance was read at 340 nm in the spectrophotometer.

Sample measuring

3ml OPA reagent were measured to a test tube and 400µl of the diluted sample solution were then added and the contents were shaken for 5 seconds. After standing for exactly 2 minutes, the solution was transferred to a cuvette and its absorbance was read at 340 nm in the spectrophotometer.

Replication

Three test tubes were used for each measurement and the contents of each test tube were measured once for absorbance at 340 nm in the disposable polystyrene cuvette and discarded. The absorbances of the three determinations were then averaged to increase accuracy

3.2.11.3 Calculation of DH

$$DH = \frac{number\ of\ peptide\ bonds\ cleaved}{total\ number\ of\ peptide\ bonds} x100\% \qquad \textit{Equation 9}$$

$$= \frac{h}{htot} * 100\% \qquad \textit{Equation 10}$$

Determination of h

$$h = \frac{((serine{-}NH2){-}\beta)}{\alpha}\frac{meqv}{g}\ protein \qquad \textit{Equation 11}$$

$$serine - NH2 = \left(\frac{Asample{-}Ablank}{Astandard{-}Ablank}\right) * \frac{0.9516meqv}{L} * 0.01 * \frac{100}{XP} \qquad \textit{Equation 12}$$

Where; P is the percentage substrate (protein) concentration in sample, 0.01 is the sample volume in litres (10ml), X is the mass of sample diluted in 10ml, A_{sample} is the sample absorbance, A_{blank} is the control absorbance (distilled water + OPA) and $A_{standar}$ is the standard absorbance (serine + OPA) and constants ($\alpha = 1$, $\beta = 0.4$, $h_{tot} = 8.6$) (Nielsen *et al.*, 2001)

3.3 RESULTS

3.3.1 Reaction with the enzyme SEBDigest F59P

Table 9-1 in appendix 9.2.1 shows the observed DH values at different combinations of the independent variables. The table shows that a highest DH of 17.4% was achieved under conditions of 55°C temperature and a pH of 8. The E/S at this peak value was 0.05%. The ANOVA table (Table 9-2) shows the significance of the influence of each of the three investigated hydrolysis factors and the following conclusions can be made from the table;

- All factors' linear terms had significant influence on the DH at 95% confidence level ($P<0.05$).
- The quadratic terms for temperature and pH were significant at 95% ($P < 0.05$) whilst the E/S quadratic term was insignificant ($P > 0.05$).
- The interaction effect of E/S and temperature was the only one with a significant effect on DH at 95% confidence interval ($P< 0.05$) whilst the other two interaction terms were insignificant ($P> 0.05$).

3.3.1.1 The optimum values

The desirability profile (Figure 3-6) predicted by the fitted model indicates that an optimum DH was achieved with a hydrolysis temperature of 60°C, pH of substrate at 7.6 and an E/S level of 0.05%. The DH increased as the temperature was increased from 30°C, and reached a peak value at 60°C, beyond which any further increase resulted in a decrease in the DH. For pH, the highest DH was located at pH 7.6, and increasing the pH beyond 7.6 also lowered the DH. The E/S gave a highest DH at 0.05%. The DH significantly dropped as the E/S was raised to levels higher than 0.05%. Therefore the optimum values predicted by the fitted model (Equation 12) were a pH of 7.6, a temperature of 60°C and an E/S ratio of 0.05% (w/w) substrate content.

3.3.1.2 The model equation

From the regression coefficients table (Table 9-3), the best descriptive model equation is as follows;

$$Y = -114 - 36.32x_1 + 38.7x_1^2 + 0.91x_2 - 0.006x_2^2 + 27.7x_3 - 1.79x_3^2 - 1.2x_1x_2 + 7.7x_1x_3 - 0.02x_2x_3 \qquad \textit{Equation 13}$$

Where Y is the DH (%), x_1 is the enzyme concentration, E/S (%w/w), x_2 is the temperature (^{o}C) and x_3 is the pH.

3.3.1.3 Model validation

Results from comparisons of model predicted and observed validation runs are displayed in Table 9-4. The residuals variation is small, ranging from 0.6 to 1.3. Figure 3-5 shows the relationship between the DH values predicted by the model and the observed values from the validation runs graphically. A correlation coefficient (R) of 0.97 suggests the existence of a strong relationship between the values. The coefficient of determination is also high (0.95). The small p - value ($p < 0.05$) further confirms that the correlation between the observed and predicted values is substantially different from zero.

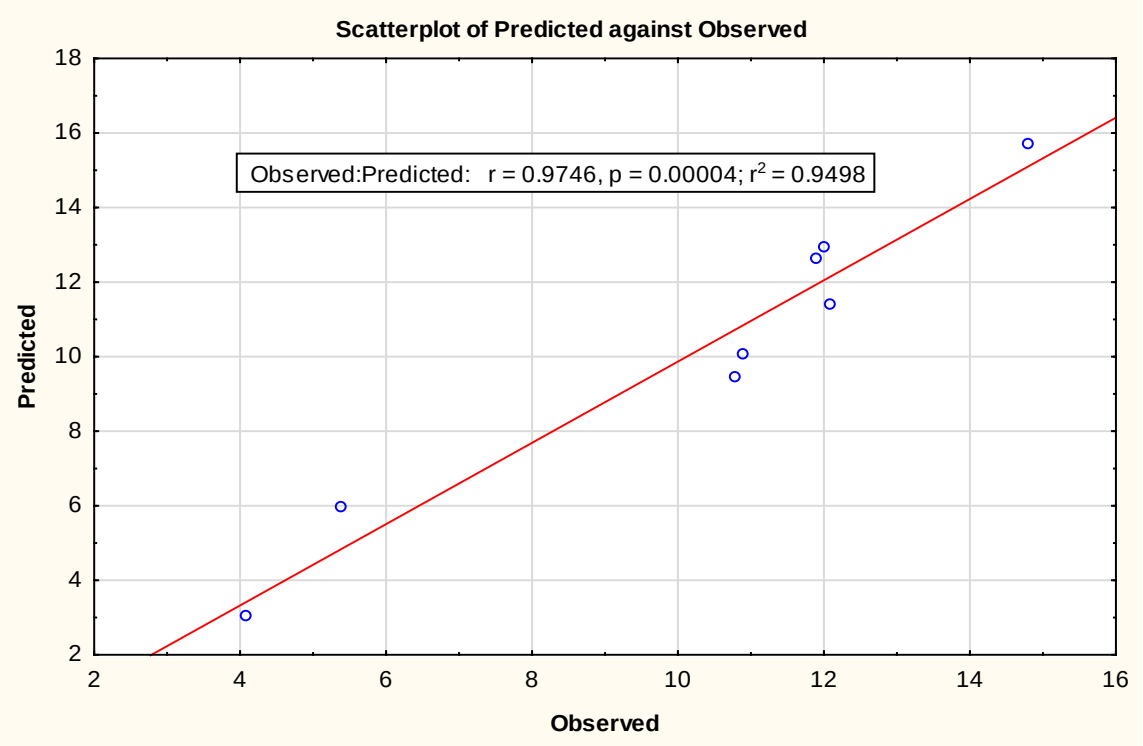

Figure 3-5; Predicted against observed DH values for model validation

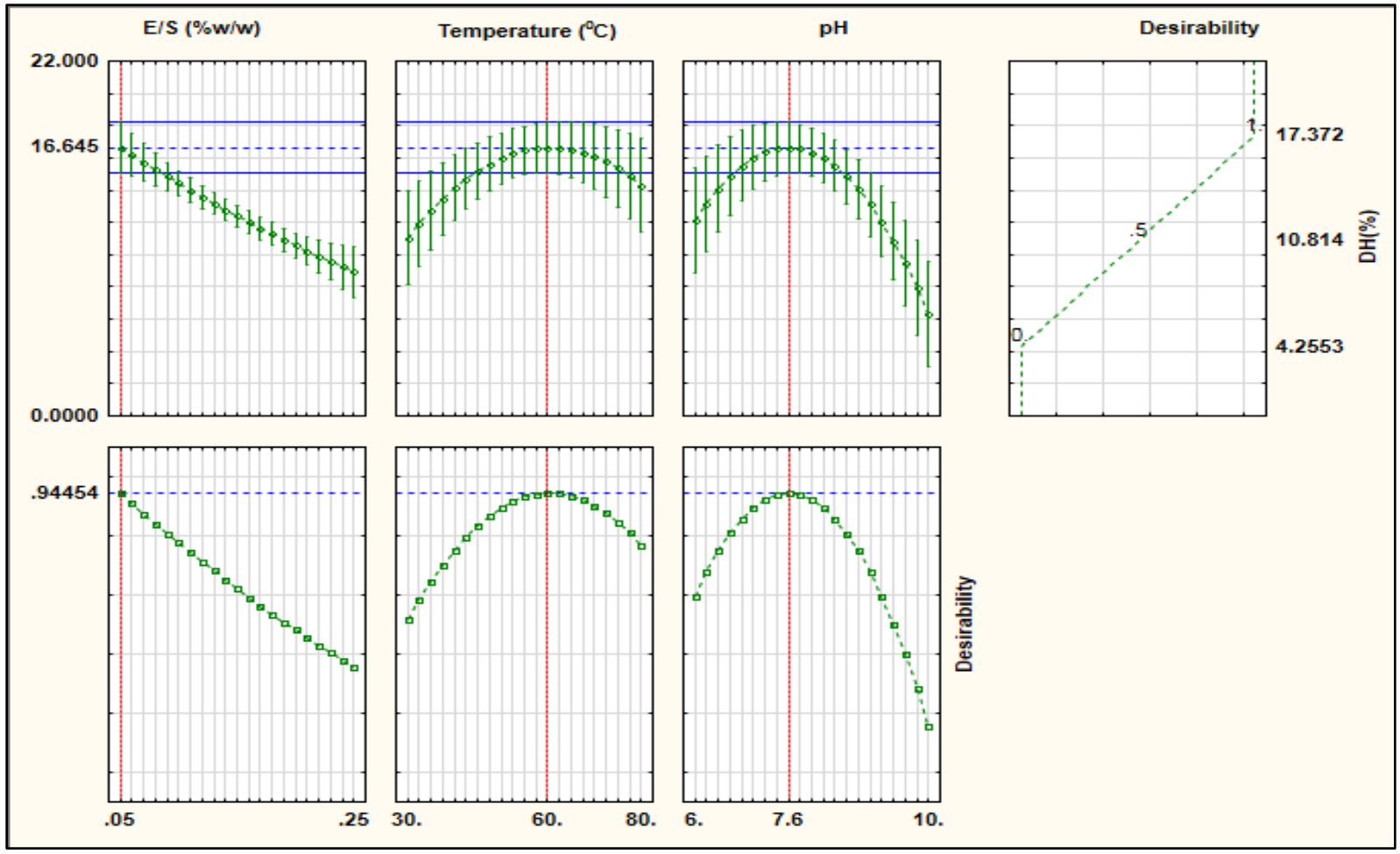

Figure 3-6; Desirability profiles for DH as affected by temperature, E/S and pH

3.3.2 Reaction with the enzyme SEBPro XL

The resultant values of the DH at various factor levels are shown on Table 9-4. A highest DH of 23.1% was achieved with SEBPro XL after an hour's hydrolysis. The highest DH was at a pH of 7 and a temperature of 55°C, with an E/S at 0.7%.

From the ANOVA table (Table 9-5), the following conclusions about the three independent factors are made;

- All the linear and quadratic terms for the three factors were significant at 95% confidence level ($P < 0.05$).
- The interaction effects between E/S and temperature, as well as that between E/S and pH had a significant effect on the DH at 95% confidence interval ($P< 0.05$).
- The interaction effect between pH and temperature was not significant at 95% confidence level ($P > 0.05$).

3.3.2.1 The optimum values

Figure 3-6 is the desirability profile of independent variables. The profile indicates that an optimum DH was achieved with a hydrolysis temperature of 60°C, substrate pH of 6.9 and an E/S level of 0.67%. The DH and desirability levels increased as the three variables were raised up to the optimum levels and decreased with further increases above the optimum conditions.

3.3.2.2 The model equation

From the regression coefficients table (Table 9-10), the best descriptive model equation for the data is as follows;

$$Y = -106.65 + 26.80x_1 - 2.18x_1^2 + 0.80x_2 - 0.005x_2^2 + 44.61x_3 - 44.03{x_3}^2 + 4.1x_1x_3 - 0.24x_2x_3 \quad \textit{Equation 14}$$

Where Y is the DH (%), x_1 is the pH, x_2 is the temperature (°C) and x_3 is the enzyme concentration, E/S (%w/w).

3.3.2.3 Model validation

The DH values obtained from validation runs for the optimisation of the hydrolysis reaction using SEBPro XL are displayed on Table 9-8 in appendix 9.2.2. The variation between the observed and predicted values is also small, ranging from 0.6 to 1.4. The relationship between the values is shown on Figure 3-7, with a correlation coefficient of 0.98 and a coefficient of determination of 0.95. The p-value is small ($p < 0.05$) confirming that the correlation between the observed and predicted values is substantially different from zero.

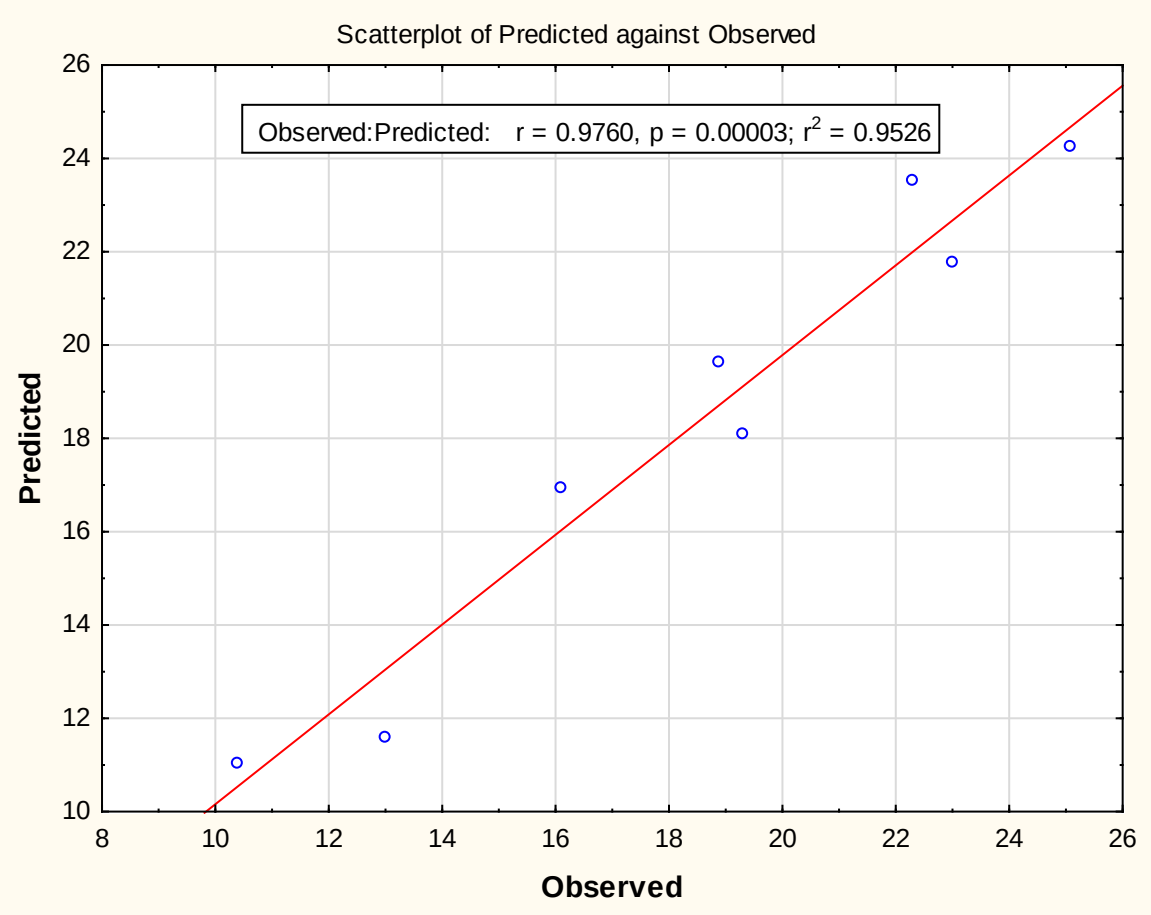

Figure 3-7; Predicted against observed values DH for model validation

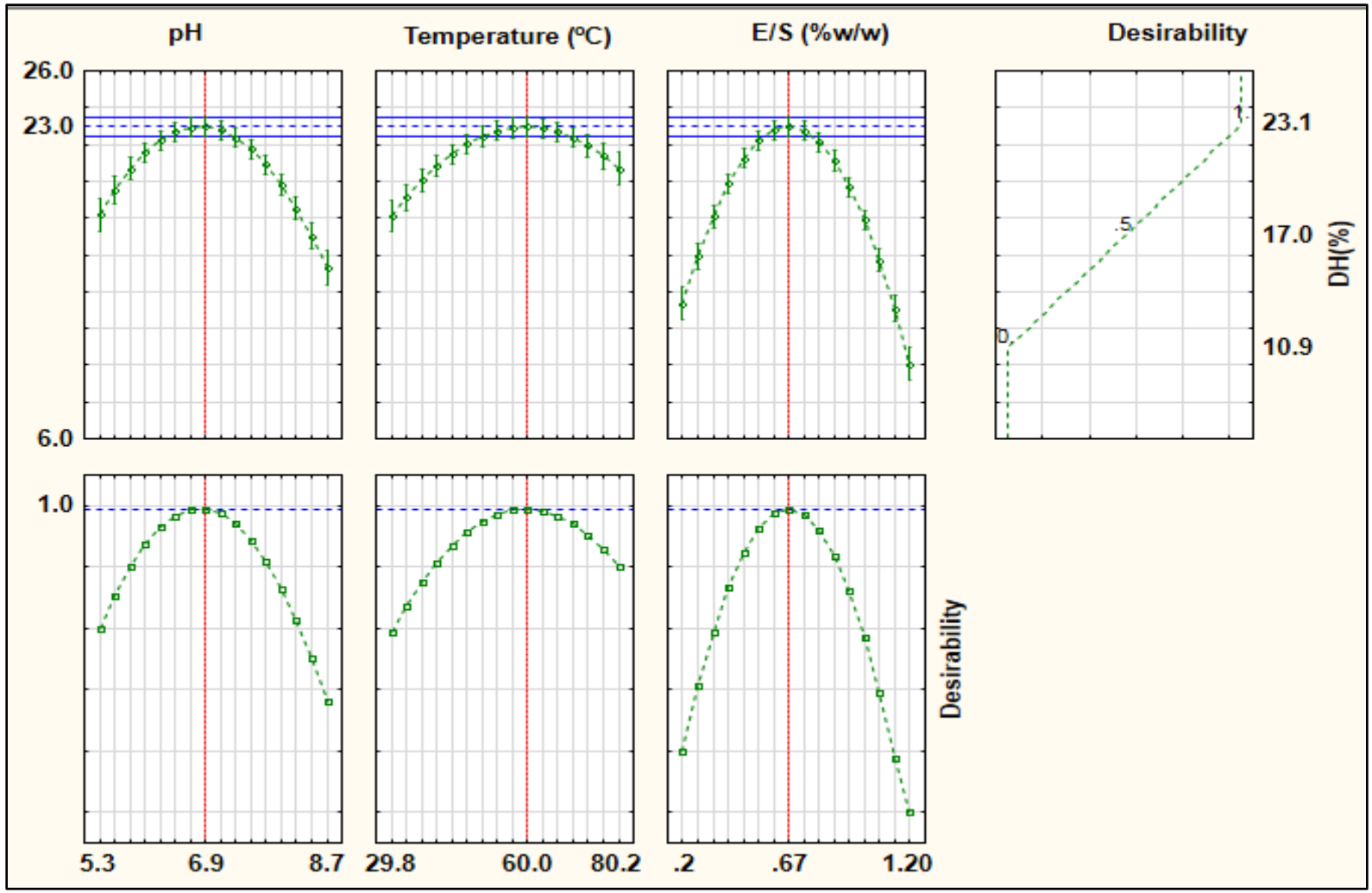

Figure 3-8; Desirability profiles for DH as affected by temperature, E/S and pH

3.4 DISCUSSION

Analysis of variance (ANOVA) results for the two enzymes confirm that the investigated factors (temperature, pH and E/S) contributed to the response, which is the DH (Tables 9-2 and 9-6). The high adjusted coefficients of determination (R^2 = 0.93 and 0.98) imply that 93% and 98% of the behaviour variations of hydrolysis with SEBDigest F59P and SEBPro XL respectively could be explained by the fitted models, and thus confirming the accuracies of the fitted models.

The influence of these three hydrolysis factors agrees with the findings of a number of previous researchers, e.g. Adler Nissen (Adler-Nissen, 1976) and Benjakul and Morrissey (Benjakul & Morrissey, 1997). Adler-Nissen (Adler-Nissen, 1976) investigated the hydrolysis of soy protein by bacterial proteases and reported the significant influence of hydrolysis conditions on the cleavage of the peptide bond. Diniz and Martin (Diniz & Martin, 1996) and Bhaskar and colleagues (Bhaskar *et al.*, 2008) also reported similar results. The model results further concur with the findings of Diniz and Martin (Diniz & Martin, 1996) for SEBPro XL, who reported significant influence of all linear and quadratic terms on the DH. For SEBDigest F59P, the quadratic effect for enzyme concentration was insignificant.

Figure 9-5 and 9-8 are response surface graphs to predict the behaviour of the DH as influenced by temperature and enzyme concentration. The graphs show that temperature and enzyme concentration, interactively affect DH. DH is affected negatively by the increase in E/S from 0.05% to 0.25% for SEBDigest F59P. For SEBPro XL, the DH increased to the highest value (0.67% E/S on figure 3-8) and decreased at E/S ratios below and above 0.67%. This decrease of DH above the optimum could be due to enzyme inhibition, or the enzyme hydrolysing itself (Diniz & Martin, 1996). The negative response in DH to increases in E/S were also reported by Guerard (Guerard *et al.*, 2001), Ovissipour and colleagues (Ovissipour *et al.*, 2010), as well as Wilkinson (Wilkinson, 1971). When studying the hydrolysis of Pacific whiting solid waste with Alcalase, Benjakul and Morrissey (Benjakul & Morrissey, 1997) reported that although E/S ratios higher than the optima increase the DH, the rate of increase in DH per unit E/S ratio is reduced.

From the desirability profiles (Figures 3-6 and 3-8), and response surface graphs (Figures 9-5, 9-6, 9-8 and 9-9), the DH increases with temperature up to 60^{o}C for both enzymes, then starts dropping with further increases in temperature. The reason for such a trend is that initially as the

temperature increases, it results in more collisions between the substrate and the enzymes, thus increasing formation of enzyme substrate complexes, which increases the DH. However, above a certain limit the enzyme becomes denatured, thereby reducing its biological activity (Bisswanger, 2002; Marangoni, 2003; Ovissipour *et al.*, 2009) and hence a reduction in DH.

The combined effects of temperature and pH as presented on figures 9-6 and 9-9 for SEBDigest F59P and SEBPro XL respectively show that the optimum DH for both enzymes is centrally positioned on the graphs, confirming that the optimum values are within the experimental region (Bezerra *et al.*, 2008). As with temperature, the degree of hydrolysis rises with an increase in pH up to an optimum levels (7.6 and 6.9 for SEBDigest F59P and SEBPro XL respectively), then decreases with further increases in the substrate pH. The decrease is caused by the enzymes denaturing above certain pH regions, thus reducing the enzymes biological activity, and therefore the DH (Bugg, 2004; Ovissipour *et al.*, 2010; Robinson, 2015).

The high coefficients of determination ($R^2 = 0.95$), high correlation coefficients ($R = 0.97$) and small p-values ($p < 0.05$) for both models validation runs (Figures 3-5 and 3-7) confirm the suitability of the fitted models to estimate the experimental values. These high values of regression and correlation coefficients (close to 1.0) emphasise the validity of the prediction models for mathematically describing the hydrolysis process (Bhaskar *et al.*, 2008).

3.5 CONCLUSION

From the results obtained in the study, the following conclusions are made;

- The DH was significantly influenced by the hydrolysis conditions that included temperature, pH of the substrate and the E/S ratio.
- The hydrolysis conditions for obtaining the optimum DH using SEBDigest F59P were a temperature of 60 ^{o}C, pH of substrate at 7.6 and an E/S ratio of 0.05% of substrate concentration.
- The hydrolysis conditions for obtaining the optimum DH using SEBPro XL were a temperature of 60 ^{o}C, pH of substrate at 6.9 and an E/S ratio of 0.67%.

These optimum hydrolysis conditions were considered for the next phase (phase two) of the study.

4. PHASE 2: PROCESS DEVELOPMENT

4.1 INTRODUCTION

Having optimised the process conditions, the next step was to evaluate the optimal production schedule to maximize DH, using different combinations of the two enzymes. Five sets of enzyme treatments were designed and employed in a series of hydrolysis reactions. Their reactions were analysed based on DH, amino acid (AA) profiles, micro mineral profiles and free amino acid (FAA) concentration. The enzyme treatment with the most favourable results with respect to DH and FAA was selected for hydrolysing rainbow trout by-products.

The following were the different enzyme treatments considered for selection;

- A reaction catalysed entirely by SEBPro XL.
- A reaction catalysed entirely by SEBDigest F59P
- A reaction initiated by SEBPro XL, with SEBDigest F59P added after 90 minutes of the hydrolysis reaction, Combination treatment1 (CT1).
- A reaction initiated by SEBDigest F59P with SEBPro XL added after 90 minutes of the hydrolysis reaction, Combination treatment 2 (CT2).
- A reaction in which the two enzymes were both added simultaneously at the beginning of the hydrolysis reaction, combination treatment 3 (CT3

4.2 MATERIALS AND METHODS

4.2.1 Hydrolysis with SEBPro XL

The reaction was run at the optimum process conditions of SEBPro XL, a temperature of 60°C, pH of 6.9 and E/S of 0.67%. 200g of rainbow trout by-products were suspended in 200g deionised water, a ratio of 1; 1 and hydrolysed for three hours with SEBPro XL. Aliquots (50ml) of the hydrolysis mixture were withdrawn every thirty minutes and deactivated, acidified, centrifuged and then analysed, as described in section 3.2.6.4.

4.2.2 Hydrolysis with SEBDigest F59P

The optimum conditions for the enzyme employed for this reaction were a temperature of 60°C, pH of 7.6 and E/S of 0.05% substrate protein concentration. The hydrolysis process was conducted as described in previous sections.

4.2.3 Hydrolysis with enzyme combinations

In order to harness the digestive nature of both enzymes, one being an endo-protease and the other exo-protease (SEBDigest F59P and SEBPro XL respectively), they had to be used together in combinations (Nilsang *et al.*, 2004; Pasupuleti & Braun, 2010). Three combination treatments were designed. In two of the reactions, one of the enzymes had to be added first, with the other being added 90 minutes into the reaction. In the third reaction, the two enzymes were both added at the initial stage of the reaction. The reaction conditions for the three combination treatments are tabulated in Table 4-1.

Table 4-1; Enzyme treatments reaction conditions

Enzyme treatment	Initiating Enzyme (Charged at t = t_o)				Intermediary Enzyme (Charged at t = t_{90})			
	Enzyme name	pH	Temperature (oC)	E/S (%w/w)	Enzyme name	pH	Temperature (oC)	E/S (%w/w)
SEBPro XL	SEBPro XL	6.9	60	0.67	n/a	n/a	n/a	n/a
SEBDigest F59P	SEBDigest F59P	7.6	60	0.05	n/a	n/a	n/a	n/a
CT1	SEBPro XL	6.90	60	0.67	SEBDigest F59P	7.6	60	0.05
CT2	SEBDigest F59P	7.60	60	0.05	SEBPro XL	6.9	60	0.67
CT3	SEBPro XL & SEBDigest F59P	7.25	60	0.72	n/a	n/a	n/a	n/a

Where t_o indicates the commencement of the reaction and t_{90} refers t_o 90 minutes of the hydrolysis reaction

4.2.3.1 Combination treatment 1; SEBPro XL first

In this reaction, the enzyme SEBPro XL was the hydrolysis initiator. The initial process conditions were a pH of 6.9 and a temperature of 60^{o}C (Table 4-1). An optimum dosage of the enzyme (0.67%) was added to start the reaction and allowed to run for 90 minutes with sample withdrawal at thirty minute intervals. The second enzyme, SEBDigest F59P was added (0.05%) after the withdrawal of the third sample at ninety minutes and the pH of the hydrolysis mixture had been adjusted to 7.6 by adding 4N KOH. The reaction was then left to run the last 90 minutes and a further three samples collected. After deactivation, acidification and centrifugation, the samples were also analysed for the DH, AA, FAA and micro mineral profiling.

4.2.3.2 Combination treatment 2; SEBDigest F59 first

The hydrolysis conditions for combination treatment 2 are as shown on Table 4-1. In this reaction, the temperature was set at 60^{o}C, being the common optimum for the two enzymes. The initial pH was set at 7.6 and SEBDigest F59P (0.05%) was added to initiate the reaction. At 90 minutes, the pH was adjusted by addition of 4N KOH to 6.9, the optimum for SEBPro XL. A percentage of 0.67% substrate concentration of SEBPro XL was then added to catalyse the remaining 90 minutes of the reaction. Samples were also withdrawn at thirty minute intervals and analysed.

4.2.3.3 Combination treatment 3; both enzymes added at the beginning

In this reaction, both SEBPro XL (0.67%) and SEBDigest F59P (0.05%) were added to the reaction mixture to initiate the hydrolysis (Table 4-1). The temperature was set at 60^{o}C, the optimum for both enzymes, but the pH was averaged to 7.25. Optimum concentrations for both enzymes were simultaneously added as the reaction began. The hydrolysis was run for 180 minutes with samples withdrawn after every 30 minutes for analysis.

4.2.4 Determination of the DH

The DH was analysed by the OPA method as described in section 3.2.11.

4.2.5 Amino acids profiling

The amino acid analysis was done by ARC – Irene Analytical services laboratories. The method used for the analysis of amino acids involved acid hydrolysis, pre-column derivatisation, separation by HPLC and detection using a fluorescence detector (Einarsson *et al.*, 1983).

4.2.5.1 Tryptophan analysis

Whereas the other aromatic amino acids can be determined by acid hydrolysis, Trp is completely destroyed by the method (Fountoulakis & Lahm, 1998). Therefore this amino acid was determined through a spectrophotometric method as described by Jiaoyan Ren, 2006 (Jiaoyan Ren *et al.*, 2006).

Chemicals

Analytical grade chemicals including diphenylamine sulphonic acid sodium (DSAS), sodium nitrite, sulphamic acid and sodium acetate, were supplied by Sigma-Aldrich, South Africa whilst sodium hydroxide, sulphuric acid and ethanol were supplied by Lasec, South Africa.

Reagent preparation

Deionised water was used to prepare solutions of sodium nitrite (1 mol/l), DSAS (2g/l), sulphamic acid (25g/l) and sulphuric acid (2mol/l). The solutions were stored in a refrigerator and used within one week.

Standard procedure

3ml of DSAS solution were measured into a set of three 40ml glass bottles. The glass bottles were then placed in an ice bath and kept at 5 °C. 6ml of sulphuric acid were then added into each bottle, shaken gently and left for 5 minutes. 2.5ml of sodium nitrite solution were added to each bottle and the bottles were placed in the ice bath for a further 5 minutes. After the 5 minutes, 3ml of the sulphamic acid solution were also added and the bottles were cooled with occasional shaking for another 5 minutes. The sample solutions were then added, 5ml per bottle and then the mixture was made up to 25ml with sulphuric acid and shaken thoroughly. 3ml of the mixture from each bottle were then transferred to a cuvette (3ml disposable polystyrene type), and incubated for 15 minutes before the absorbance was read at 522 nm in a spectrophotometer. The sample Trp concentration was calculated using Beer's Law (Equation 15) and the Trp standard curve (Figure 9-12, appendix 9.5). Linearity of the curve was confirmed in the concentration range of 0.3 – 12 µg/ml of tryptophan.

$$A = \varepsilon . b . c \qquad \textit{Equation 15}$$

Where; A is the sample absorbance, b is the length of the path travelled by light through the sample (cm), c is the concentration (mol/L) and ε is a molar absorptivity (8900 L/mol.cm) constant that depends on both wavelength and substance.

4.2.6 Free amino acids

Free amino acids were determined using an ultra-performance liquid chromatography (UPLC) separation with fluorescence detection after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). The analysis was done by the Central Analytical Facility at the University of Stellenbosch, under the supervision of Mr. Malcom Taylor. 1µl of sample/standard solution was injected into the mobile phase which conveys the derivatised amino acids onto a Waters UltraTag C_{18} column (2.1 x 50mm x 1.7µm) held at 60°C. Analytes eluting off the column were detected by a personal digital assistant (PDA) detector, with each amino acid coming off the column at a unique retention time. Instrument control and data acquisition was performed by Mass Lynx software which integrates the peaks at the defined retention times and plots calibration curves for each amino acid based on the peak response (peak area/internal standard peak area) against concentration.

4.2.7 Mineral profile analysis

The inductively coupled plasma-mass spectrometry (ICP-MS) analysis was used to analyse the mineral content of the fish by-products and FPH in wet weight basis at the Central Analytical facility (CAF) laboratory at the University of Stellenbosch under the supervision of Ms. Riana Rossouw. The instrument was calibrated using NIST (National Institute of Standards and Technology, Gaithersburg MD, USA) traceable standards to quantify selected elements. All the elements except calcium and selenium were analysed under He-collision mode to remove polyatomic interferences. Calcium and selenium were analysed under H-reaction mode. Two types of samples were sent for micro element analysis, the minced fish processing by-product sample and the clear, aqueous FPH samples which had been acidified to pH 3 by phosphoric acid. Only the solid fish processing by-product sample was acid digested prior to analysis.

4.2.8 Statistical analysis

The data produced in the experimental runs were analysed with Microsoft excel and the STATISTICA software. The correlations between the DH and FAA obtained by utilising different enzyme treatments were obtained by Pearson's correlation coefficient (R) which evaluates the strength of two variables' relationship. The (R^2) values and the p-values (with significant differences for $p < 0.05$) were calculated by multiple regression analysis and t-tests respectively using the STATISTICA software.

4.3 RESULTS

4.3.1 The Degree of Hydrolysis

The observed DH values for the five enzyme treatments over three hour hydrolysis reactions are displayed on Table 9-9 in appendix 9-3 whilst the graphical presentation of the hydrolysis progress curves is shown on figure 4-1.

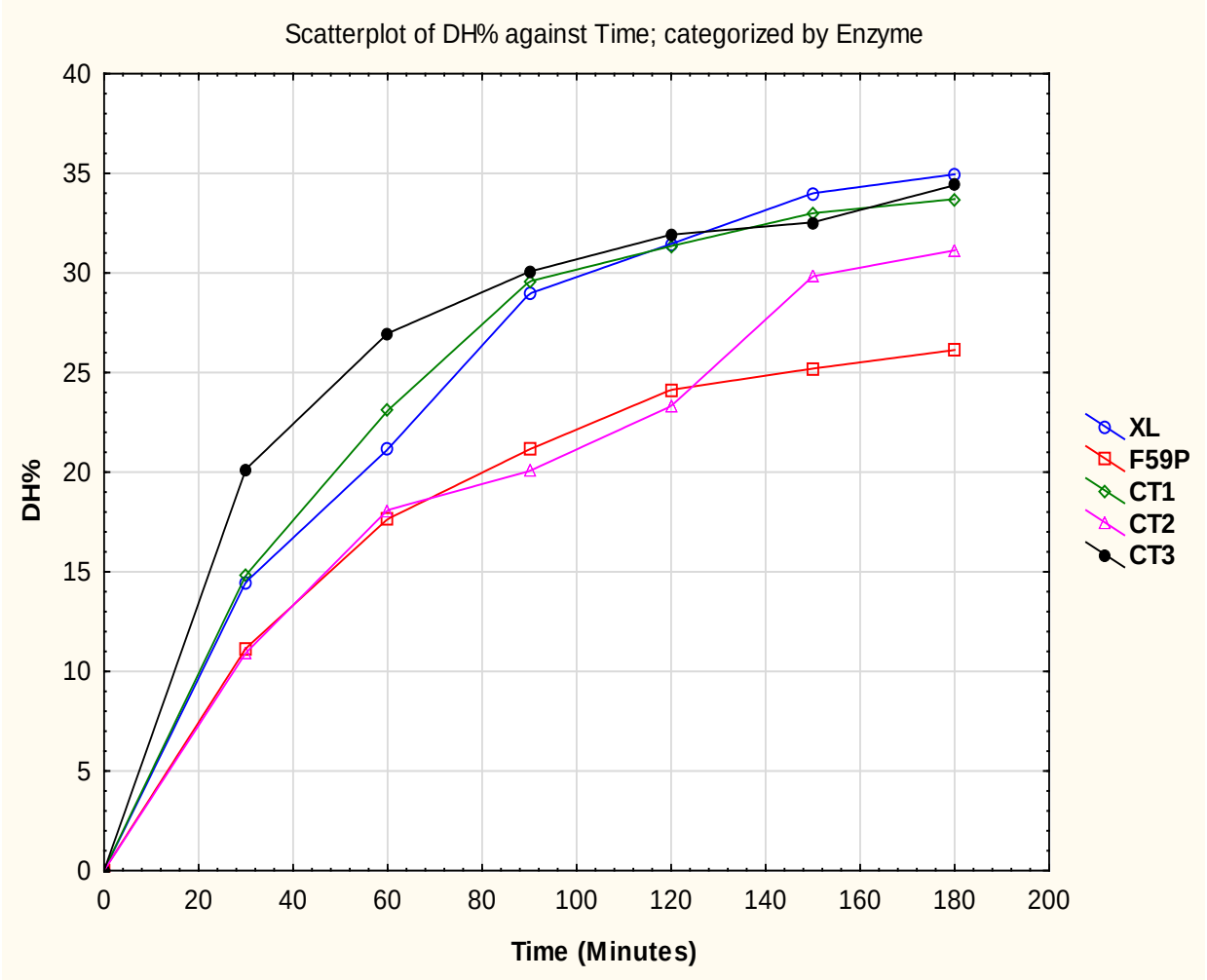

Figure 4-1; Hydrolysis curves for hydrolysis with all the five enzyme treatments

The graph (Figure 4-1) shows that of the five enzyme treatments, SEBPro XL attained the highest DH, followed by CT3 and then CT1. CT2 had the fourth whilst SEBDigest F59P had the least DH. The differences in DH of the top three enzyme treatments are however very small, (35% for

SEBPro XL, 34.4% for CT3 and 33.7% for CT1). The hydrolysis curves are similar in shape for all the FPH, showing high rates of hydrolysis at the reaction initiation that slow down as the reaction progresses. The graph shows that CT3 had the highest initial rate of hydrolysis. It reached a DH of 20% in the first 30 minutes and 27% after 60 minutes of hydrolysis. The treatment however quickly reached its convergence phase (at 90 minutes), managing a small increase of 4.45% in DH in the last 90 minutes of the hydrolysis reaction. It was 0.6% lower than SEBPro XL after the scheduled 3 hours of reaction.

The enzyme treatment CT1 did not show any effect of the addition of the second enzyme, whilst CT2 showed an upward trend at the 120th minute of reaction after the addition of the second enzyme. The increase in the DH for CT2 was lower between 60 and 90 minutes (2%) than between 90 and 120 minutes (3.2%), further increasing to 6.5% between 120 and 150 minutes. This showed that the reaction was converging under the catalysis of SEBDigest F59P, but when SEBPro XL was added, the reaction rate increased. Thus addition of SEBPro XL as a second enzyme increased the rate of hydrolysis, and the DH.

4.3.2 Mineral profiles

The mineral composition of the FPH catalysed by the different enzyme treatments for 3 hours are presented in Table 9-10 in appendix 9-4. The profiles of macronutrients, micronutrients and harmful elements in the FPH are presented in the succeeding subsections.

4.3.2.1 Macronutrients

The assayed macro nutrients for the FPH hydrolysed by the five enzyme treatments are presented in table 9-10 and Figure 4-2. From figure 4-2, it can be concluded that phosphorous was the most abundant nutrient in FPH of all the enzyme treatments (55g/l in SEBPro XL, 42.5g/l in SEBDigest F59P, 44g/l in CT1, 42g/l in CT2 and 60.5g/l in CT3). The enzyme treatment CT3 catalysed FPH contained more macro nutrients than the rest of the treatments, with the most plenteous being phosphorous, whilst magnesium was the least (< 1000ppm) in all FPH.

Potassium concentration was also higher in the CT3 catalysed FPH (15068 ppm) than the rest of the treatments, whilst the SEBPro XL catalysed FPH had the least abundant potassium (9536 ppm).

The concentration of potassium in the remaining treatments did not differ greatly and ranged from 10602ppm in SEBDigest F59P to 11826ppm in CT2, whilst CT1 had 11134ppm.

Calcium concentration was high in CT3 (17058 ppm) and very low in all the other treatments (<300ppm) whilst magnesium concentration was low in all the five treatments (< 1000ppm). Total sulphur and nitrogen were not analysed.

4.3.2.2 Micronutrients

The micronutrient concentrations in the five different enzyme treatments' FPH are shown on Figure 4-3. Enzyme treatment CT3 produced an FPH with the highest concentrations of zinc, which was the most abundant micronutrient (70.2ppm), and iron (50.8ppm). Boron (20ppm), copper (4.6ppm) and molybdenum (0.1ppm) were more abundant in the SEBPro XL catalysed FPH than the rest of the FPH, whilst a higher manganese concentration (6.2ppm) was recorded in the CT3 catalysed FPH than the rest of the FPH. Molybdenum content was very small in all treatments (<1 ppm). The CT3 catalysed FPH contained the highest total nutrient content (143.6 ppm), followed by SEBPro XL (95.2 ppm). CT1 contained the third most abundant micronutrient (77ppm) whilst CT2 (68.7 ppm) and SEBDigest F59P (67.6 ppm) contained the fourth and fifth abundant contents of micronutrient respectively.

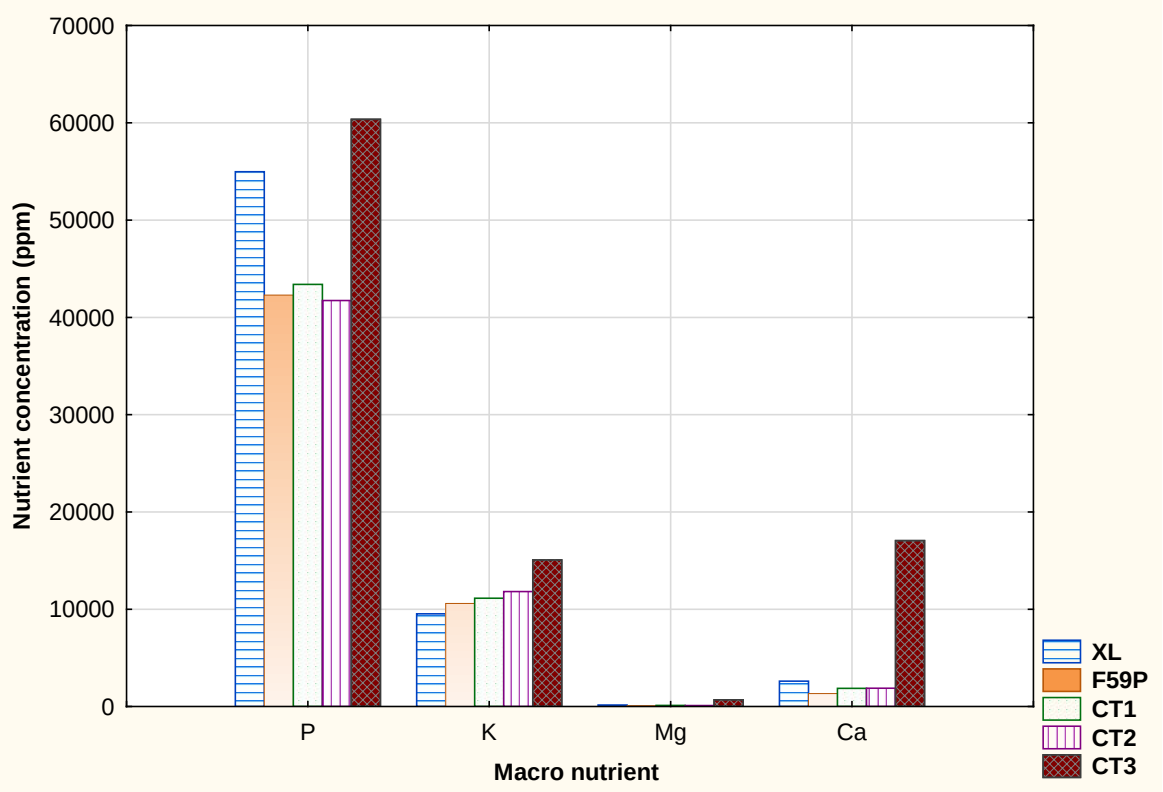

Figure 4-2; Macronutrient content in FPH of different enzyme treatment

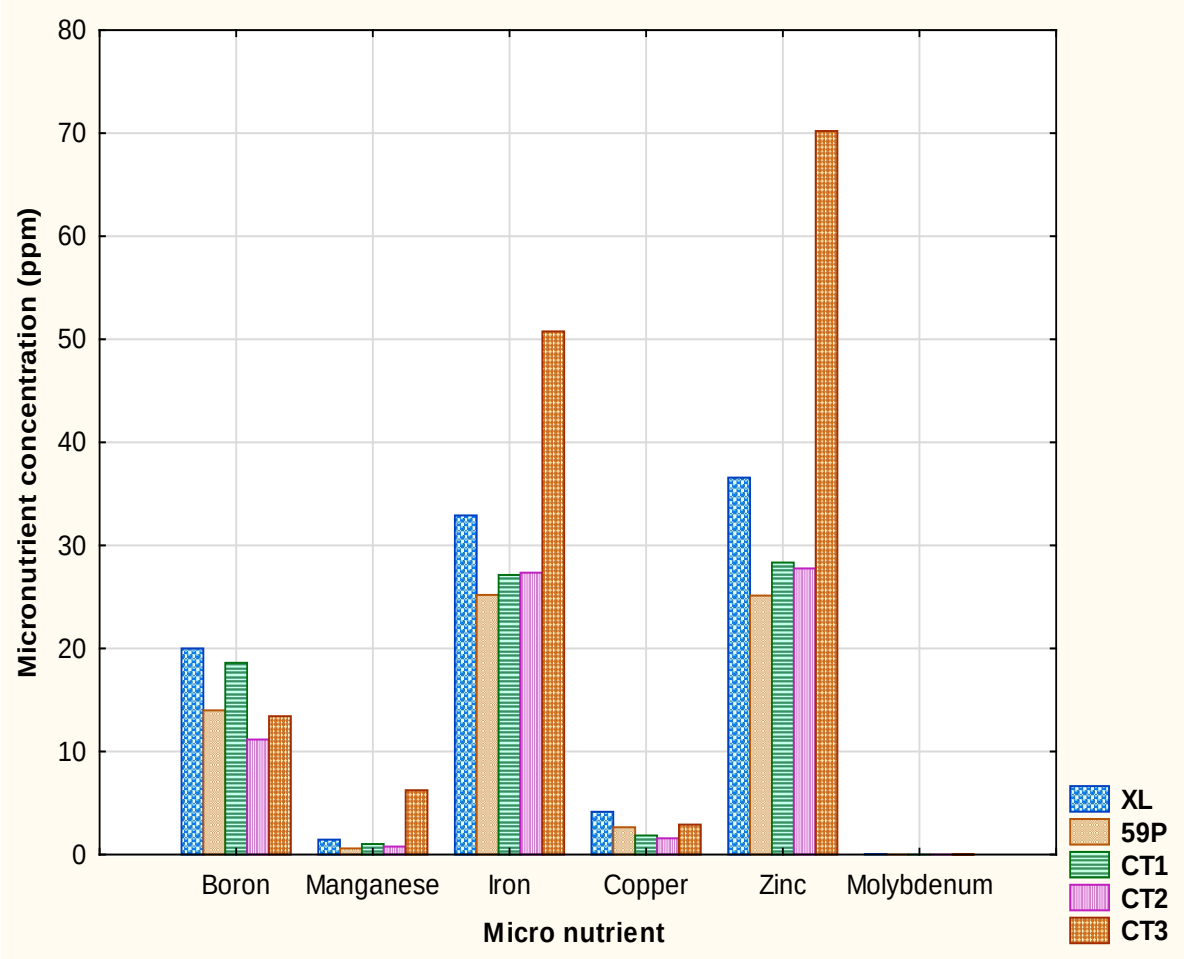

Figure 4-3; Micronutrients content in FPH of different enzyme treatments

4.3.2.3 Harmful elements

Table 4-2 shows the content of micronutrients with potential harmful effects if applied above certain limits. The second column lists the legislative gazetted limits. As is evident from the table, all FPH's harmful elements quantities were within the acceptable levels.

Table 4-2; Maximum allowable and actual concentrations of harmful elements in different FPH

Elements	Maximum allowable	XL	F59P	CT1	CT2	CT3
	Concentration (ppm)					
Cd	20	0.02	0.01	0.01	0.01	0.02
Cr	1750	0.05	0.06	0.06	0.06	0.06
Cu	750	4.16	2.66	1.85	1.58	2.91
Hg	10	0.01	0.01	0.01	0	0.01
Ni	200	0.04	0.03	0.03	0.03	0.1
Pb	200	0.03	0.02	0.04	0.03	0.03
Zn	2750	36.58	25.14	28.34	27.77	70.21
As	20	1.72	1.71	1.7	1.59	1.71
Se	15	0.2	0.17	0.18	0.13	0.25

4.3.3 Amino acids

The amino acid compositions of the five FPH are presented on Table 9-11 in appendix 9-5. The table shows that all amino acids had higher concentrations in the SEBPro XL catalysed FPH than the others. Three of the enzyme treatments (SEBDigest F59P, CT1 and CT2) completely destroyed methionine. Glycine in the SEBPro XL catalysed FPH had the highest concentration of all amino acids (37464ppm).

4.3.3.1 Total amino acids

Figure 4-4 is a bar graph of FPH total amino acids in the FPH catalysed by the five enzyme treatments. The graph depicts the FPH hydrolysed by enzyme treatment SEBPro XL as the one with the highest content of total amino acids (24.9%), followed by CT3 (7.2%) and then CT1 with 4.6% , and SEBDigest F59P with 4.5 % whereas CT2 had the least content of amino acids (3.9%).

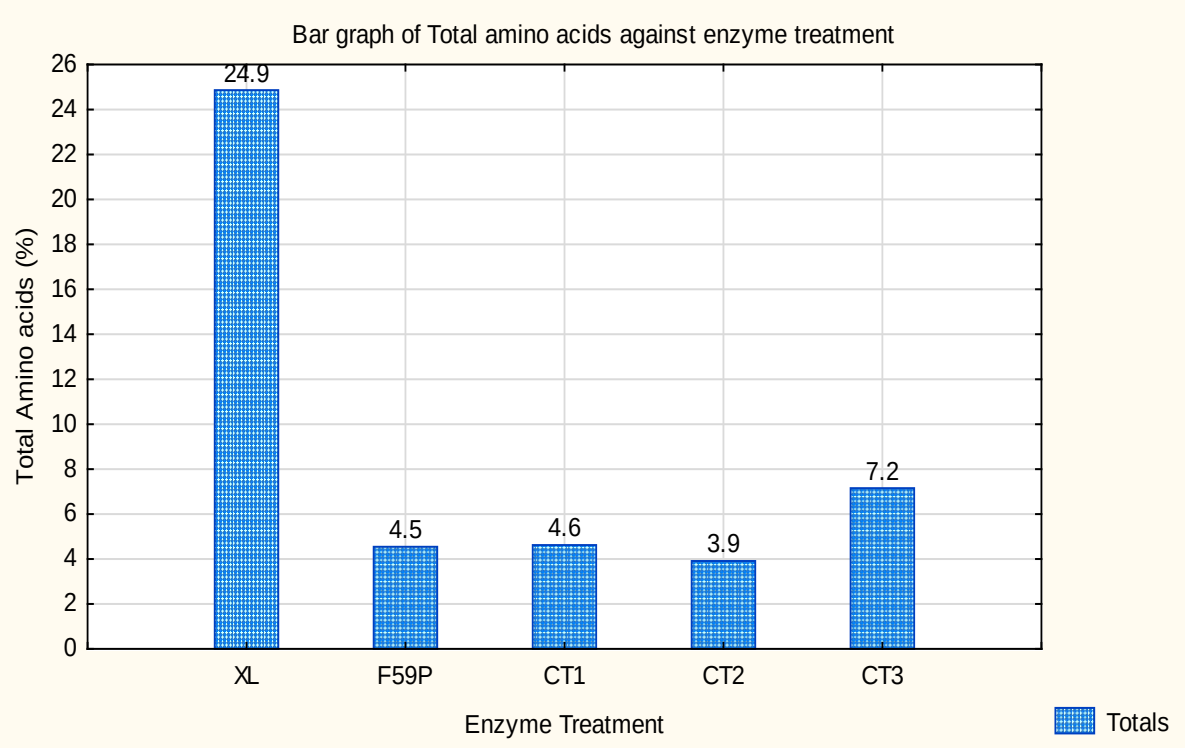

Figure 4-4; Total amino acids in FPH of the five enzyme treatments after 3 hours of hydrolysis

4.3.4 Free Amino Acids

The progress curve results for the total FAA in the different FPH catalysed by the five enzyme treatments are presented on Figure 4-5. Table 9-12 (in appendix 9-6) shows the compositions of FAA in the different FPH.

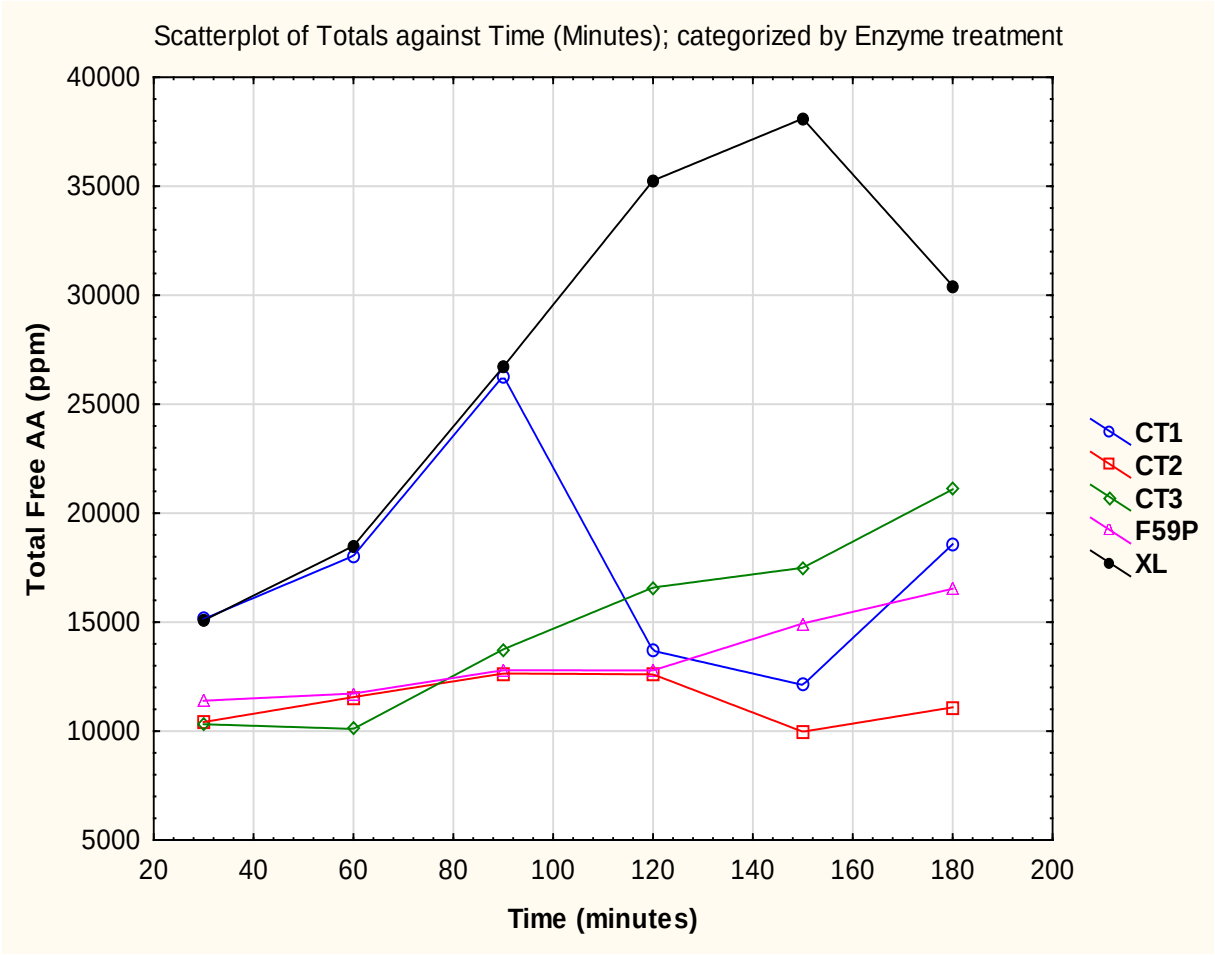

Figure 4-5; Behaviour of total free amino acids with time for the five enzyme treatments

The graph shows that the SEBPro XL catalysed FPH had the highest concentration of FAA in each sample. The FAA content rose with time from 15000ppm at 30 minutes until the 150th minute where it reached its peak (381085 ppm). Increasing hydrolysis time further than 150 minutes saw the FAA content drop to 304029 ppm at reaction termination at 180 minutes. However, at that level it was still significantly higher than CT3 which was the second highest. CT3 FAA content gradually increased from 10314 ppm at 30 minutes to end the reaction after 180 minutes at 21093

ppm. CT1 FAA content rose up till the 90th minute, peaking at 26298 ppm, before significantly dropping at 120 minutes to 13685 ppm. It dropped further at 150 minutes to 12116 ppm before rising to end the reaction (180 minutes) at 18572 ppm. SEBDigest F59 had a gradual increase in FAA content as well, though the values were very low (11394 at the start and 16531ppm at the end of reaction). It gave the lowest concentrations of FAA. The CT2 FAA content had a gradual rise from 10000ppm at 30 minutes to attain 15000ppm after 120 minutes. The FAA content then dropped to 10000ppm at the 120th minute interval but rose again to end the reaction at 11500ppm after 180 minutes.

4.3.4.1 Catalysing with SEBPro XL

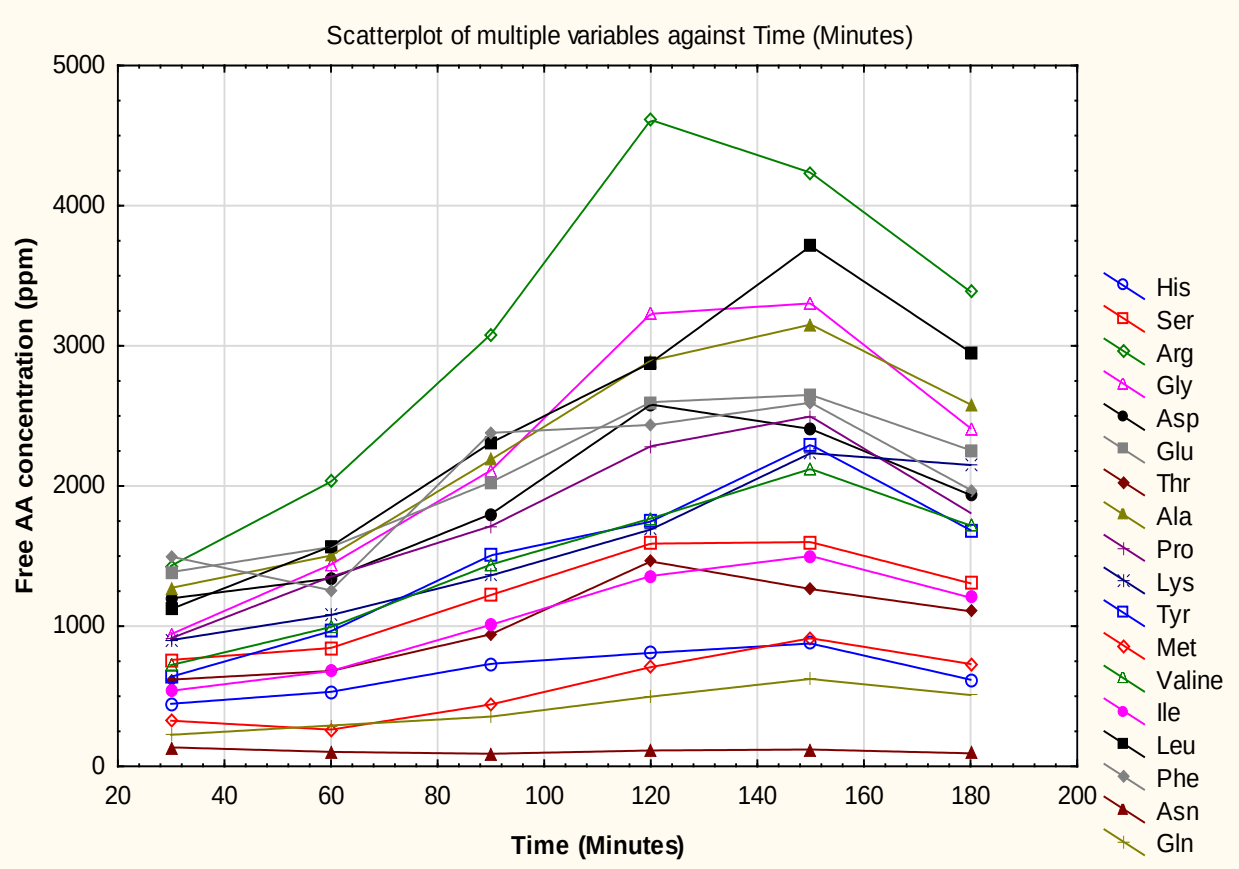

Figure 4-6; Behaviour of FAA with time in a SEBPro XL catalysed FPH

Table 9-13 in appendix 9-6 shows the compositions of FAA in the FPH catalysed by SEBPro XL with time. The progress curves graphically representing the same FPH are shown on Figure 4-6. Arginine had the highest concentration in the FPH (3386 ppm), followed by leucine (2954 ppm) and then alanine (2581 ppm).

The FAA rose to their maximum values between 120 minute and 150 minute periods. They then dropped as the reaction progressed. This trend is portrayed by all FAA in the SEBPro XL catalysed FPH (Figure 4-6), except asparagine, which itself had very low values (at most 135 ppm). Asparagine amounted to 135 ppm after the first 30 minutes of the reaction and slowly dropped along the hydrolysis progress to end the reaction at 93 ppm. Arginine, asparagine, glycine, glutamic acid, serine and threonine started dropping after 120 minutes of the hydrolysis reaction, whilst the rest dropped after 150 minutes.

4.3.4.2 Catalysing with SEBDigest F59P

The FAA compositions in the SEBDigest F59P catalysed reaction FPH are displayed on Table 9-14 in appendix 9-6. The trends are further graphically presented on Figure 4-7. The graph shows that Glycine was the highest in the final FPH concentration at reaction termination (1565 ppm) whilst the lowest was glutamine (75.5 ppm).

Three groups are identifiable from Figure 4-7. The individual FAA concentration in the groups, although different, showed similarity in their trends as the reaction progressed. The top group, comprising of leucine, arginine, glycine, alanine, asparagine and glutamic acid initially had higher concentrations than the rest, and increased their concentrations more steeply than the other two groups. The second group, the middle group, includes valine, tyrosine, serine, isoleucine and threonine. These FAA had higher initial concentrations than the bottom group, but their concentration increases with time ware less rapid than the top group. Lastly, the bottom group, which include such FAA as histidine, asparagine, glutamine and methionine initially had low concentrations, and they deteriorated with the reaction progress, ending at lower concentrations than the initial.

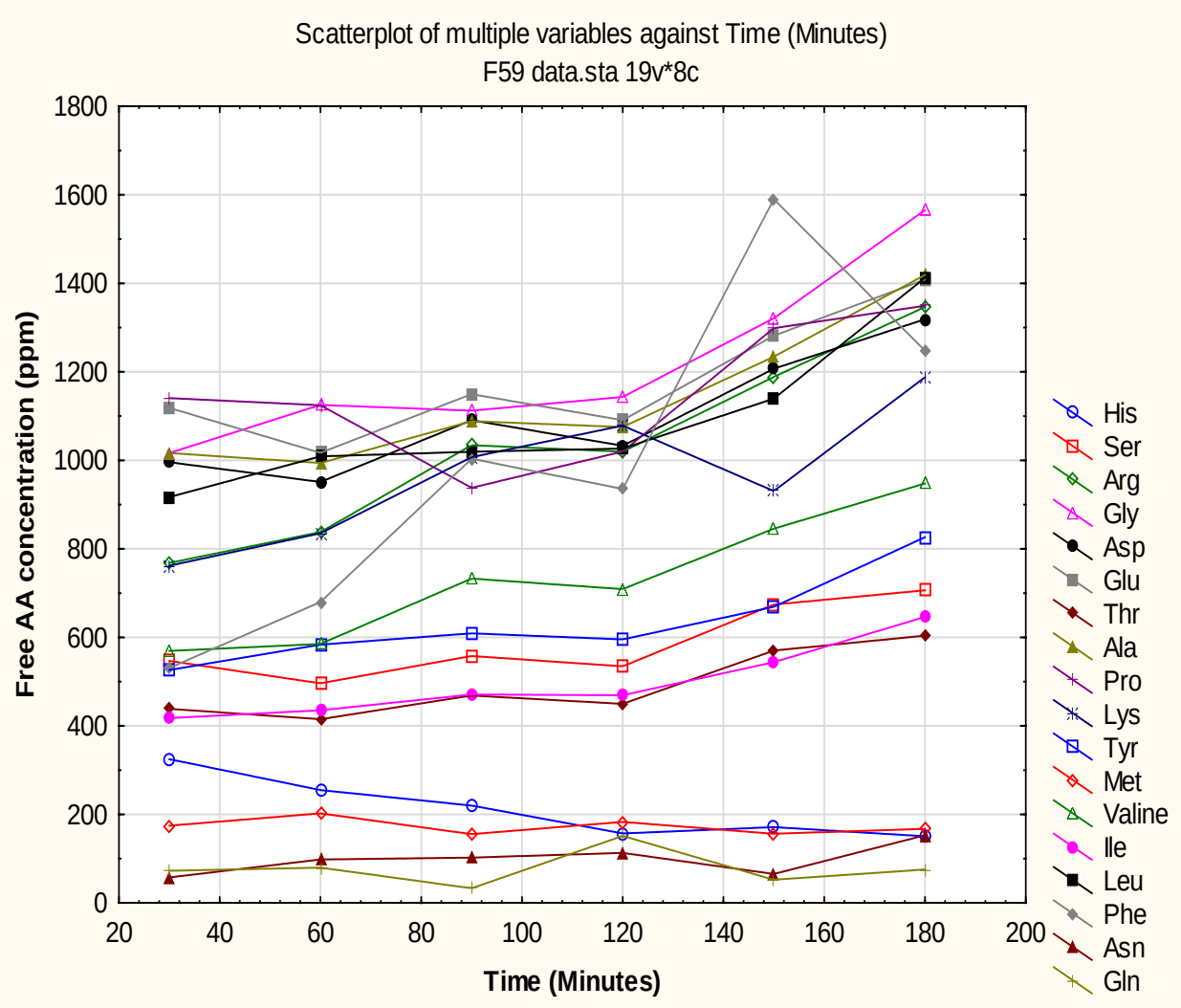

Figure 4-7; Behaviour of FAA with time in a SEBDigest F59P FPH

4.3.4.3 Catalysing with Enzyme combination treatment 1 (CT1)

The FAA composition of the FPH catalysed by the enzyme treatment CT1 are displayed in Table 9-15, and progress curves on Figure 4-8. The general trend shown by the FAA was to increase in concentration until the 90th minute when the second enzyme, SEBDigest F59 was added. They then went through two successive drops at the 120th and 150th minute marks. The first drop being more rapid than the second. After the 150th minute the concentrations started to rise again, ending the reaction at 180 minutes with positive slopes. However, the asparagine content slowly decreased as the reaction progressed. Methionine and glutamine content also decreased with reaction time

from 323ppm, to end the reaction at 9ppm for methionine. Glutamine was undetectable after 180 minutes.

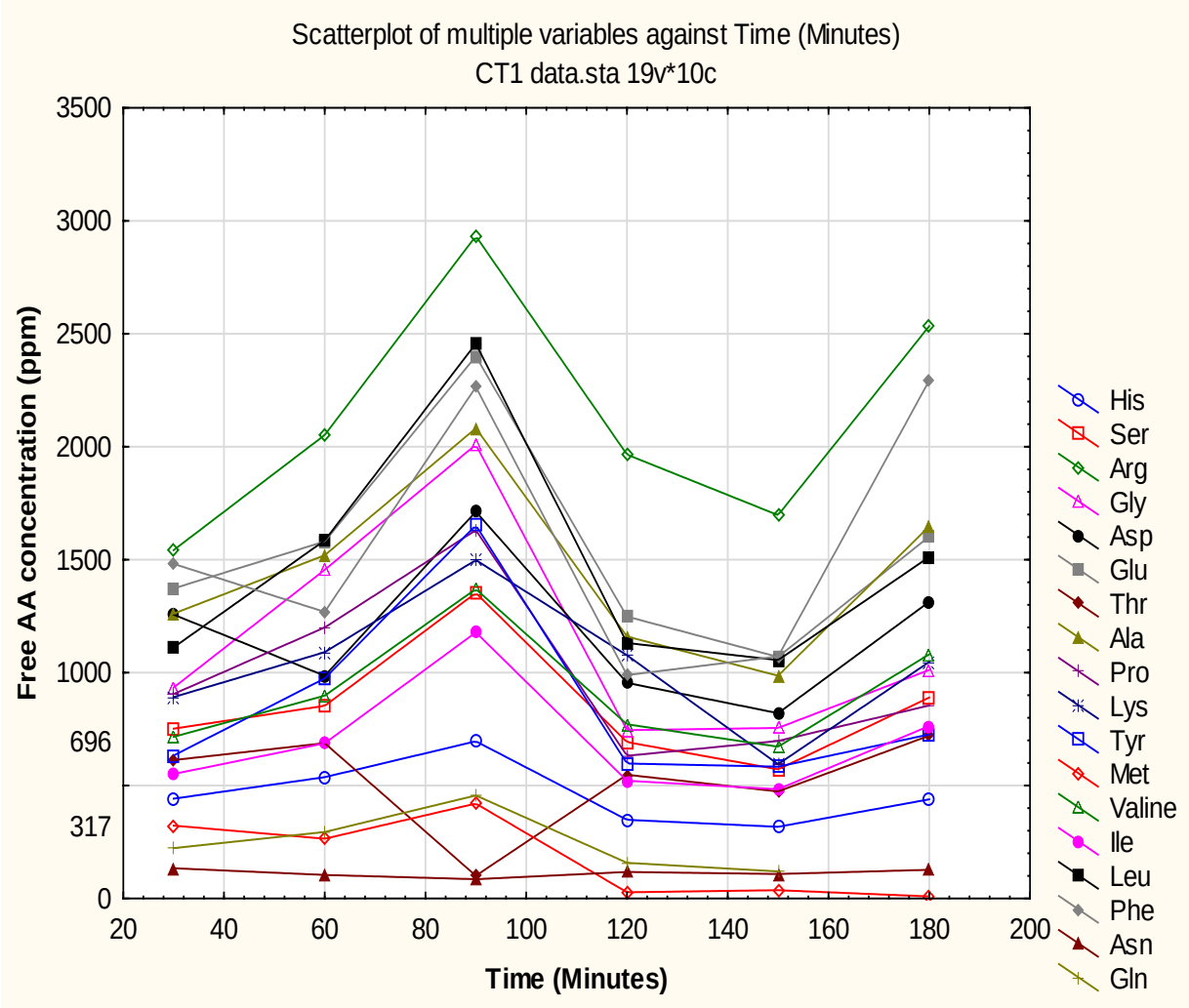

Figure 4-8; Behaviour of free amino acids with time in a CT1 FPH

4.3.4.4 Catalysing with enzyme combination treatment 2 (CT2)

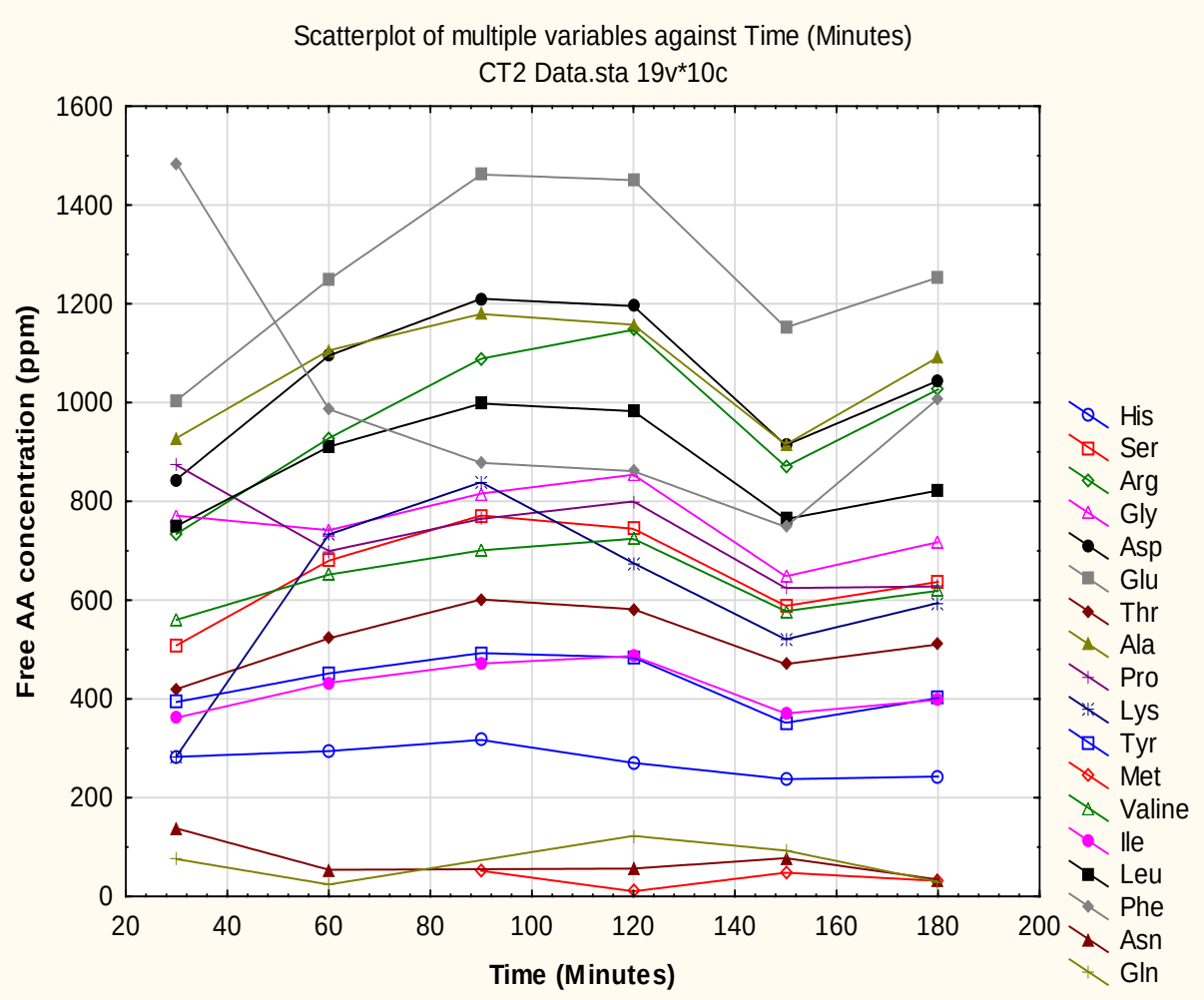

Figure 4-9; Behaviour of FAA with time in a CT2 FPH

Table 9-16 in appendix 9-6 and figure 4-9 show the FAA composition of the CT2 catalysed FPH and their progress curves with time respectively. The graph shows a generally steady increase in FAA composition with time until the 90th minute of the hydrolysis reaction. After the 90th minute, the graphs flattened, and the dropped at the 120th minute, only to start rising again. The FAAs histidine, asparagine, glutamine and methionine were the lowest in concentrations, and they progressively deteriorated as the reaction progressed.

4.3.4.5 Catalysing with Enzyme treatment combination 3 (CT3)

The FAA composition for the CT3 catalysed FPH are shown on table 9-17. The FPH's FAA progress curves are further displayed on Figure 4-10. The highest FAA concentration at reaction termination was 2397 ppm for phenylalanine and the lowest was methionine (84.8 ppm). The four FAA; asparagine, histidine, methionine and glutamine were distinctly similar in their behaviour. They had lower concentrations than other FAA, and almost remained unchanged as the reaction progressed. All other FAAs generally increased in their concentrations as the reaction progressed, except phenylalanine which, although it had the highest concentration, fluctuated along the reaction progress.

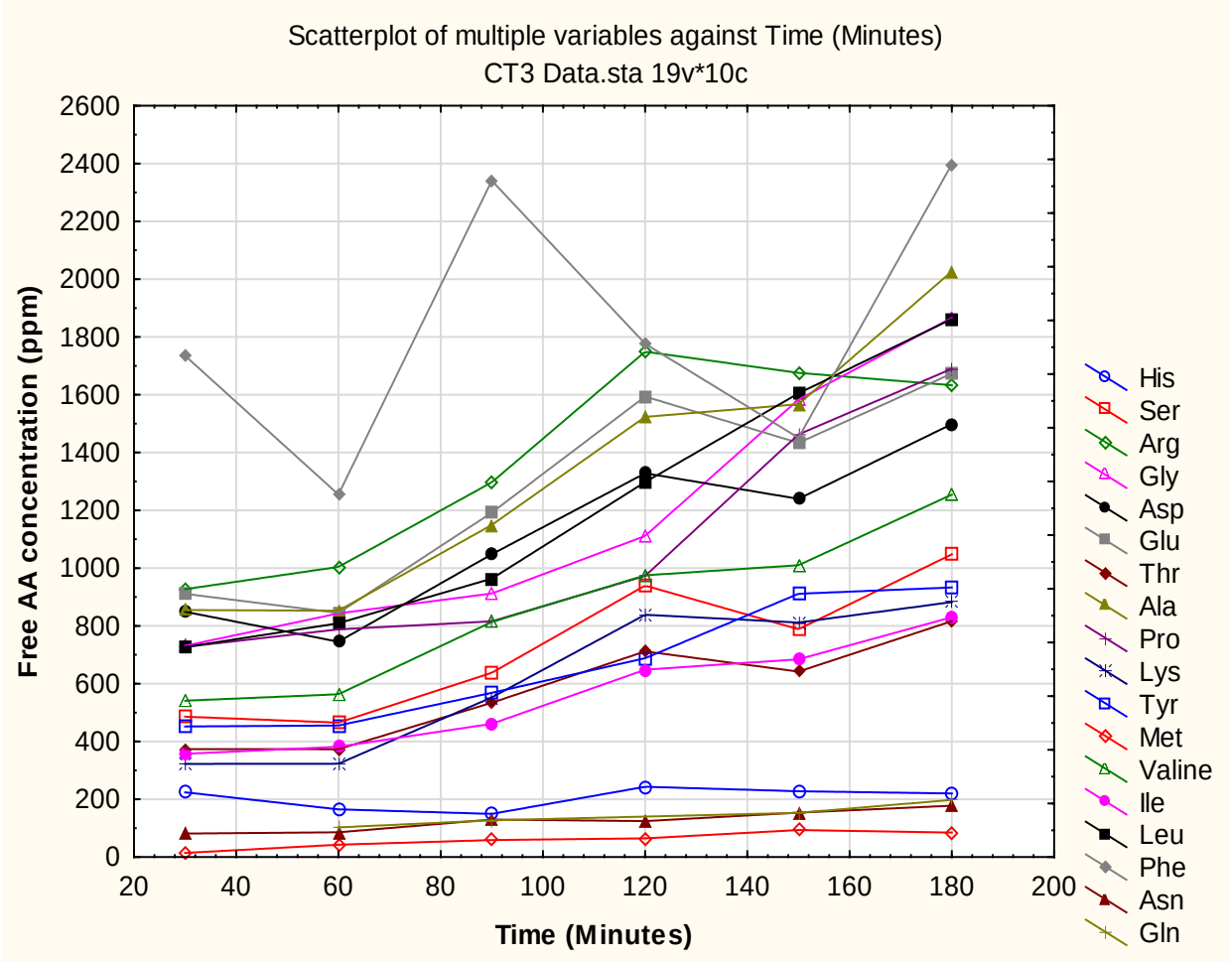

Figure 4-10; Behaviour of FAA with time in a CT3 catalysed FPH

4.3.4.6 Individual FAA progress curves

The progress curves of individual FAA with time in the five different FPH are shown on Figures 9-18 to 9-35 in appendix 9-9. Most FAA contents are higher in the SEBPro XL catalysed FPH than all the other FPH, except phenylalanine and asparagine. These two FAA did not follow particular trends as the reactions progressed but haphazardly fluctuated with each sample. Phenylalanine content was highest in CT3 (2400 ppm), followed by CT1 (2300 ppm). These high values of phenylalanine were a result of rapid rises at the 150 minute of the hydrolysis reaction. In the SEBPro XL catalysed FPH, the phenylalanine concentration rapidly fell, whilst it rose in CT3 and CT1. It thus ended the reaction with a lower phenylalanine concentration in the SEBPro XL FPH than CT3 and CT1. Asparagine content is also higher in CT3 (178 ppm) than all other FPH. The least asparagine content is in CT2 (35 ppm).

4.3.5 Correlations between degree of hydrolysis and free amino acids

Table 4-3 shows the relationships between the FAA (g/L) and DH% of FPH of the five enzyme treatments with hydrolysis progress. Graphical presentations for the correlations (Figures 9-13 to 9-17) in appendix 9-7 confirm significant correlations between the FAA and DH in the FPH catalysed by the following enzyme treatments;

- SEBPro XL ($r = 0.9693$, $p < 0.05$)
- SEBDigest F59P ($r = 0.94$, $p < 0.05$)
- Enzyme treatment CT3 ($r = 0.94$, $p < 0.05$).

The graphs further show insignificant correlations between the FAA and DH in the FPH catalysed by enzyme treatment CT1 ($r = 0.70$, $p > 0.05$) and the enzyme treatment CT2 ($r = 0.54$, $p > 0.05$).

Table 4-3; Relationship between DH (%) and FAA (g/l) for reactions with the five enzyme treatments

	SEBPro XL		SEBDigest F59P		CT1		CT2		CT3	
Time (mins)	FAA(g/L)	DH%	FAA(g/L)	DH%	FAA(g/L)	DH%	FAA(g/L)	DH%	FAA(g/L)	DH%
30	15.1	14.5	11.4	11.2	15.1	14.8	10.4	10.9	10.3	20.1
60	18.5	21.1	11.7	17.6	18.0	23.1	11.6	18.1	10.1	27.0
90	26.7	29.0	12.8	21.1	26.3	29.6	12.6	20.1	13.8	30.1
120	35.3	31.5	12.8	24.1	13.7	31.3	12.6	23.3	16.6	31.9
150	38.1	34.0	14.9	25.2	12.1	33.0	10.0	29.8	17.5	32.5
180	30.4	35.0	16.5	26.1	18.6	33.7	11.1	31.1	21.1	34.4

4.4 DISCUSSION

The shape of hydrolysis curves obtained (Figure 4-1) showed initial high reaction rates, which gradually dropped as the reactions progressed. This is consistent with the results reported by Shahidi and colleagues (Shahidi *et al.*, 1995), as well as Guerard (Guerard *et al.*, 2001), Nilsang and colleagues (Nilsang *et al.*, 2004) and Nguyen (Nguyen *et al.*, 2010) who associated the initial high rates of reaction to the high prevalence of unhydrolysed peptide bonds present being hydrolysed, and yet deplete as the hydrolysis progresses. The reaction rate also decreases with reaction progress due to enzyme denaturation, inhibition by hydrolysis products, a low solubility of peptides that compete with the unhydrolysed (Rebeca *et al.*, 1991) fish protein and the possibility of auto-digestion of the enzyme (Kristinsson & Rasco, 2000).

The addition of a second enzyme during hydrolysis with CT1 did not result in notable changes to the progress curve, however in CT2 when SEBPro XL was added as an intermediary enzyme after hydrolysis with SEBDigest F59P for 90 minutes, there was a sharp increase in DH (from 23.3 to 29.8%). These two contrasting outcomes could be as a result of the hydrolysis reaction being controlled by the available peptide bonds or the action of inhibitory peptides, which had been continuously solubilised during the hydrolysis (Guerard *et al.*, 2001).

The mineral profiles of the five FPH indicated high values of potassium and phosphorus (Figure 4-2). This is an expected result as these two minerals were used as reagents in the hydrolysis process. Potassium was added as potassium hydroxide for pH control. Its addition in the hydrolysis mixture and ultimate assay in the final FPH was affected by the initial pH of the raw material and the actual pH drop during hydrolysis. Exogenous phosphorus was added as phosphoric acid after hydrolysis in order to aid sample centrifugation and act as a preservative, whilst simultaneously adding phosphorous, which is an important macronutrient of the FPH. The differences in mineral content in the five different FPH depends on the exogenous enzyme treatments applied, with CT3 being more effective in mineral solubilisation than other evaluated treatments. This result agrees with the findings of Aspmo and colleagues who reported that although lipids, minerals and non-protein nitrogen are not hydrolysed, their solubilisation is influenced by the disintegration of the protein structure by enzyme activity (Aspmo *et al.*, 2005).

The AA profiles for the five enzyme treatment catalysed FPH show the concentration of AA in the five FPH ranging from 3.9 % for the CT2 FPH to the highest in SEBPro XL FPH (24.9%). When investigating the enzymatic hydrolysis of yellowfin tuna by-products using Protamex, Nguyen and colleagues reported that protein solubility and recovery is enhanced by high rates of proteolysis (Nguyen *et al.*, 2010), increasing with an increase in hydrolysis time and E/S up to a convergent point where further increases in time and E/S will become insignificant It also depends on the activity of the enzyme applied (Aspmo *et al.*, 2005; Ramakrishnan *et al.*, 2013). Thus the high concentration of AA in the soluble fraction of the SEBPro XL FPH demonstrates its high hydrolysing and solubilising ability when compared to the other investigated enzyme treatments. However, since SEBPro XL and CT3 DH are almost similar (35% and 34.4% respectively), the large difference in their FPH AA concentrations (17.7%) suggests a high solubility of large poly-peptides in the SEBPro XL FPH.

The complete destruction of methionine (Table 9-11) in FPH of three enzyme treatments (SEBDigest F59P, CT1 and CT2) agrees with Ian Mackie (Mackie, 1981) who, in reviewing FPH, reported that sulphur containing amino acids are susceptible to breakdown during hydrolysis. Glycine, in SEBPro XL FPH had the highest concentration of all amino acids.

The SEBPro XL progress curve for total FAA (Figure 4-5) is high above the rest of the curves. Since SEBPro XL is an exoprotease (Advanced Enzymes Technologies), this is an expected outcome, given that exoproteases act on external peptide bonds to liberate FAA (Mótyán *et al.*, 2013). SEBDigest F59P, being an endoprotease, is expected to release a mixture of peptides with varying molecular weights, with less FAA than SEBPro XL (Gupta & Khare, 2007; Vishalakshi & Dayanand, 2009). The data from this investigation therefore agree with this literature.

The FAA graphs on Figure 4-5 show a general increase in FAA concentration with the progress of the reaction, except for two treatments (CT1 and CT2), which involved adding an intermediate enzyme after 90 minutes of hydrolysis. These two combination treatments responded by dropping their FAA concentrations soon after addition of the second enzyme. The FAA concentrations would however start to rise again along the course of the reaction. According to the author's knowledge, this finding is not reported in literature. The drop in FAA concentrations could be attributed to side reactions that occur when the pH is rapidly adjusted from the optimum level of

one enzyme to another, or the occurrence of the plastein reaction (Slizyte *et al.*, 2005; Zhao *et al.*, 2015), which synthesise peptides from FAA, thereby depleting the FAA concentration. During the plastein reaction, FAA condense to form a gelatinous protein when a high concentration of FAA are maintained, which then precipitates out of the hydrolysate solution (Slizyte *et al.*, 2005). This could also explain the fall in FAA composition in the SEBPro XL catalysed reaction (Figure 4-6), CT1 catalysed reaction (Figure 4-8) and CT2 catalysed reaction (Figure 4-9).

The FPH catalysed by SEBPro XL, an exogenous enzyme, had the strongest correlation between FAA and DH ($R^2 = 0.94$) whilst the CT1 catalysed FPH had the least ($R^2 = 0.49$). CT3 had a second highest correlation ($R^2 = 0.9$), SEBDigest F59P the third ($R^2 = 0.88$) whilst CT2's correlation ($R^2 = 0.54$) was second least. Significant correlations in the SEBPro XL, SEBDigest F59P and CT3 FPH indicate that the FAA increase with an increase in DH. Morais and colleagues (Morais *et al.*, 2013), when investigating the correlation between peptide profiles and FAA reported that the strength of the correlation was influenced by the type of enzyme. This is supported by the data in that SEBPro XL, an exogenous enzyme presents the strongest correlation. This is also an expected outcome since exogenous enzymes release more FAA than poly peptides during hydrolysis. Thus as peptide bonds are cleaved (increasing DH), more FAA are released, building a strong correlation between the two.

The correlation is diluted by addition of SEBDigest F59P, a second enzyme. SEBDigest F59 P is an endogenous enzyme, which releases more poly peptides than FAA. This combination results in a higher rate of reaction, with a high DH, but since the hydrolysis produces a mixture of poly peptides and FAA, it doesn't translate directly into an increase in FAA. Thus the correlation is reduced. The two enzyme treatments in which an intermediate enzyme was added (CT1 and CT2) had a poor correlation because the addition of a second enzyme during the course of a reaction reduced the concentration of FAA. This negatively influenced the relationship.

4.5 CONCLUSIONS

The following conclusions were made after considering the results in Phase 2 of this investigation;

4.5.1 Degree of hydrolysis

The highest DH was achieved by using the enzyme treatment SEBPro XL (35 %), followed by CT3 (34.4%). The next highest enzyme treatment has a 33.1% DH (CT1), 31% (CT1) and lastly 26.1% (CT2). The degrees of hydrolysis between the two top enzyme treatments (SEBPro XL and CT3) are close (0.6% difference). This made it impossible to make a distinction between the two treatments based solely on the DH.

4.5.2 Mineral profiles

The CT3 FPH had the highest amount of all macro and micro nutrients than all the other treatments (Figures 4-2 and 4-3 respectively). However, since the choice of enzyme treatment was based on FAA and DH, this trait in CT2 may only be considered when there is need for production of FPH with higher macro and micro nutrients.

4.5.3 Amino acids

The SEBPro XL FPH had the highest total AA concentration. The total amino acid content in the SEBPro XL FPH was more than thrice the concentration in CT3, the second highest FPH (figure 4-4).

4.5.4 Free Amino Acids

The SEBPro XL FPH contained the highest concentrations of free amino acids amongst all the other enzyme treatments (Table 9-12) and Figure 4-5. This made it the most preferable enzyme treatment for the next phase of the investigation.

4.5.5 Correlation between DH and FAA

Depending on the type of enzyme used for catalysis, a correlation exists between FAA and DH. Exogenous enzymes have a stronger correlation than endogenous enzymes since their hydrolysis releases mostly FAA, so an increase in DH translates to an increase in FAA. Endogenous enzymes break the protein complexes into peptides of various sizes and less amino acids, therefore the strength of the correlations between DH and FAA in their FPH will be less than that of exogenous enzyme catalysed FPH.

4.5.6 Choice of enzyme treatment

After running a set of five hydrolysis reactions with two enzymes (SEBPro XL and SEBDigest F59P) and three possible combinations of the two candidate enzymes, analysing and comparing the resultant FPH for DH and FAA, the chosen enzyme treatment was SEBPro XL. The enzyme treatment SEBPro XL had a higher DH, FAA concentration and total AA content than the other treatments investigated. The enzyme was then selected for application in the next phase of the investigation (Phase 3).

5. PHASE 3: MAXIMISING DH

5.1 INTRODUCTION

In the previous section (section 4), the highest DH achieved by the selected enzyme treatment, SEBPro XL was 35% after a hydrolysis period of three hours. The other objective of the study was to attain a DH of at least 45%. Possible ways to attain such a high DH include either increasing hydrolysis time or increasing the E/S (Benjakul & Morrissey, 1997; Marangoni, 2003; Morais *et al.*, 2013; Slizyte *et al.*, 2005). However, increasing hydrolysis time could not be considered since the industrial partner preferred a shorter hydrolysis time (at most 4 hours), which could be managed in an 8-hour shift. Thus the investigation focused on increasing the E/S.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design

The selected enzyme treatment, SEBPro XL was applied in varying concentrations to a set of five hydrolysis reactions. The reactions were run under controlled conditions of 60^{o}C and a pH of 6.9 (the optimum conditions for the preferred treatment selected during Phase 2). The enzyme concentrations were varied from 1% to 5%, and the hydrolysis reactions were run for four hours. The lowest E/S of 1% was opted for since the DH values from the previous section, at an E/S of 0.67% could only achieve a maximum DH of 35%, and was converging after 3 hours of hydrolysis (Figure 4-1). The experimental range had five levels of E/S (1 to 5%). The hydrolysis reactions were performed at optimum conditions of temperature and pH as previously described in section 3, with samples withdrawn at 30 minute intervals and prepared for analysis.

To investigate the possibility of a linear relationship between E/S (w/w substrate concentration) and DH (%), a graph of E/S against DH was plotted in excel and correlations coefficients were evaluated. Modelling of the hydrolysis process at different conditions was done using both linear non-linear regression to enable the prediction of the DH at any given time.

5.3 RESULTS

5.3.1 The Degree of Hydrolysis

The DH values for FPH of the five different E/S are tabulated in Table 9-18, in appendix 9-8. Progress curves for the same values are also shown on Figure 5-1. Each hydrolysis profile was characterised by a high initial rate which progressively decrease tending towards a convergent value. A 1% E/S achieved a maximum DH of 38% in four hours, whereas 2% E/S could attain a 43.1% DH. A 50.9% DH was attained by an E/S of 3% whilst a 4% E/S managed a 58.2% DH and a 5% E/S gave a 60% DH in a 4 hour hydrolysis reaction.

The graph (Figure 5-1) shows that at each time interval, DH increases with increasing enzyme concentration. The 5% E/S progress curve reached a maximum DH of 60% after 210 minutes and levelled out. Although all the other curves show increasing DH at reaction termination, the rates of increase are significantly reduced with time.

5.3.2 Establishing the relationship between E/S and DH

By plotting E/S (%w/w) against DH (%), linear relationships were observed and they are displayed on Figure 5-2. From these relationships, the exact concentrations of enzymes required to hydrolyse trout by-products to the required DH, from 1 hour to 4 hours, can be determined. The graph shows that to get exactly 45% DH in a 4 hour hydrolysis period, an E/S ratio of 2.2% to substrate concentration by weight is required. 2.6% E/S and 3.4% E/S are required to achieve a DH of 45% in 3 and 2 hours respectively.

The linear graphs (Figure 5-2) had high coefficients of determination (0.97 for the 4-hour hydrolysis, 0.98 for 3-hour hydrolysis, 0.995 for the 2- hour hydrolysis and 0.96 for the 1-hour hydrolysis reactions).

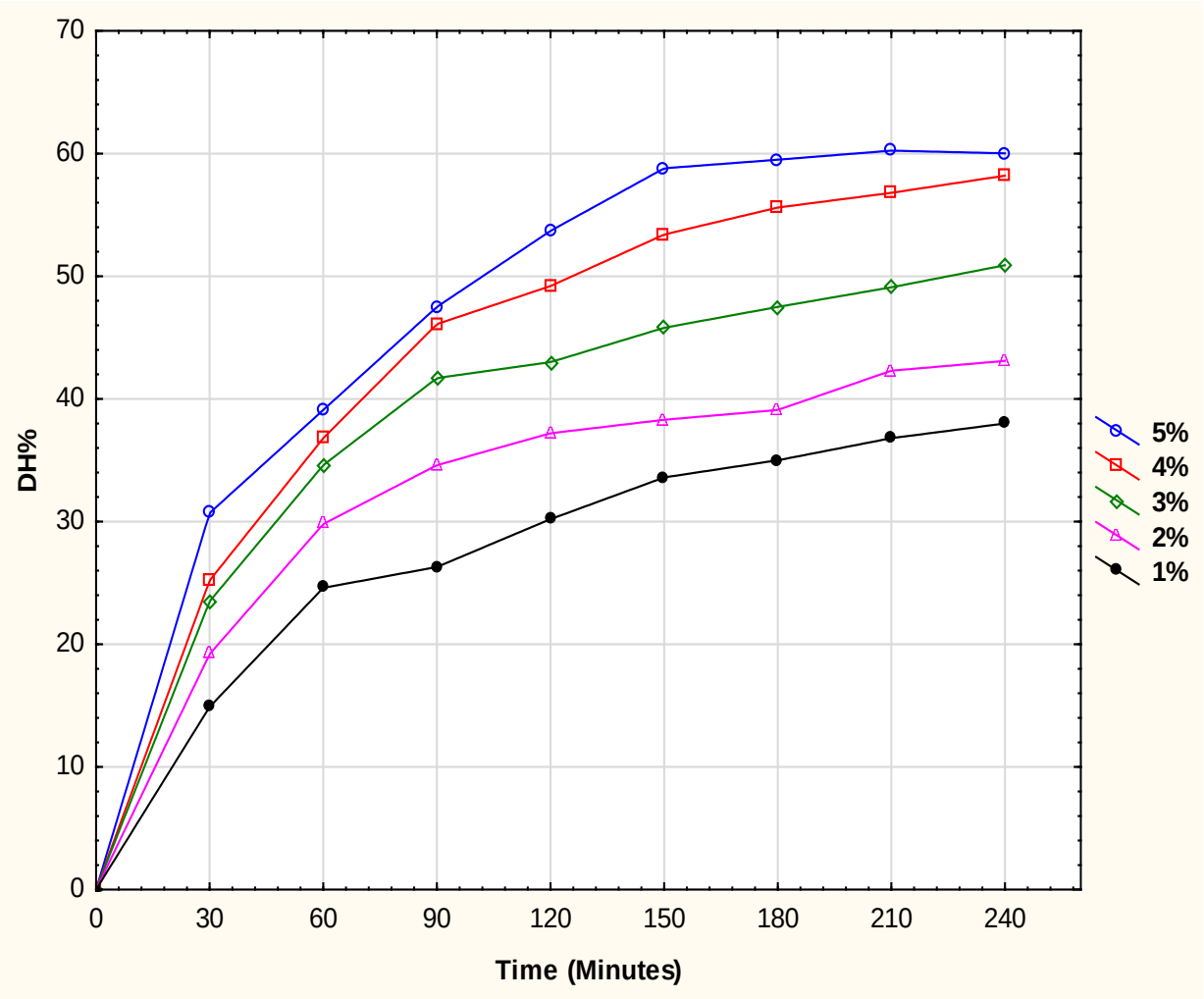

Figure 5-1; DH progress curves for varying E/S when catalysing with SEBPro XL

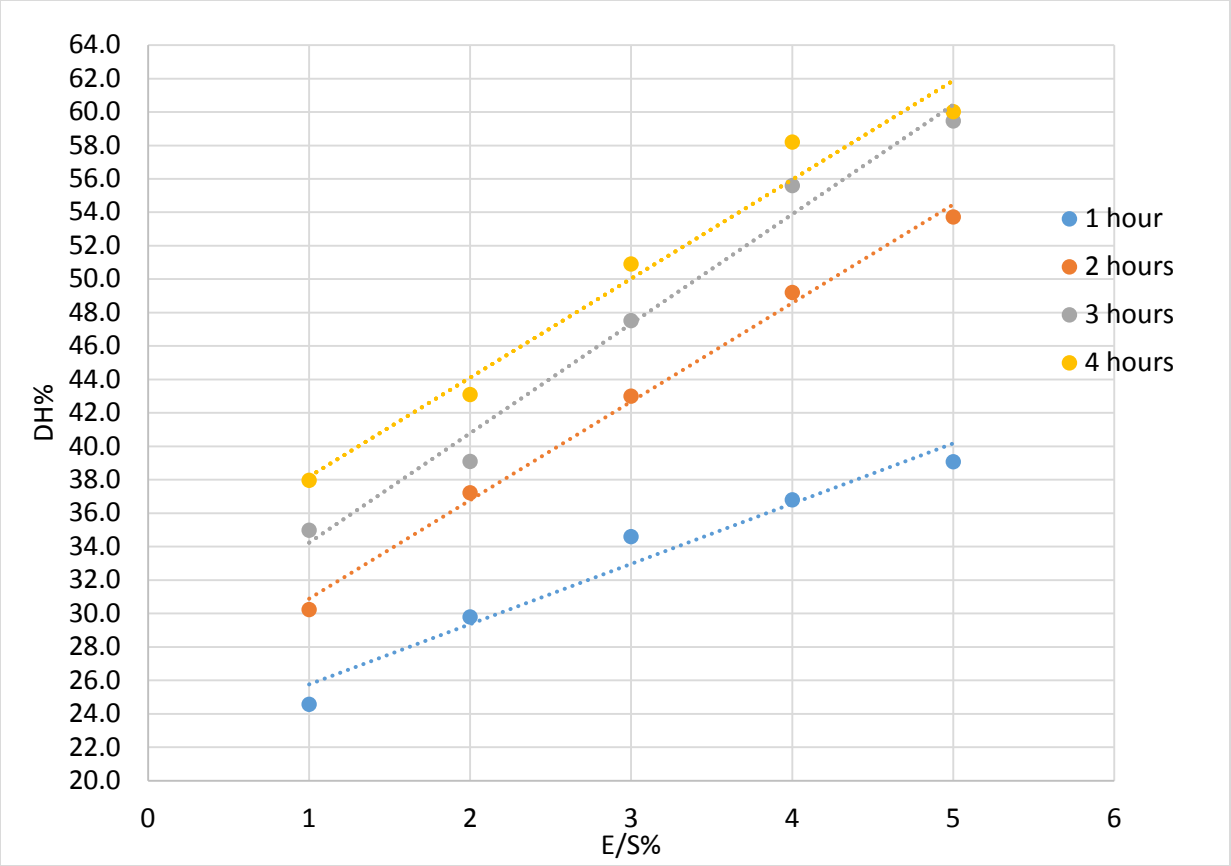

Figure 5-2; Relationship between E/S (%w/w) and DH (%) for trout heads catalysed by SEBPro XL

The linear models produced by the data to estimate the DH are given in table 5.1 for an E/S ratio of 5%, temperature of 60°C and a pH of 6.9. Y is the DH whilst *x* represents the hydrolysis time.

Table 5-1; Model equations and R^2 values for the plot of DH% against Time (min) and E/S (%)

Hydrolysis time (Hours)	Model Equation	Coefficient of determination (R^2)
1	$y = 3.6045x + 22.15$	0.9591
2	$y = 5.8954x + 24.99$	0.9952
3	$y = 6.5486x + 27.69$	0.9833
4	$y = 5.922x + 32.27$	0.9713

5.3.3 Establishing the relationship between E/S and time (minutes) at a DH of 45%

For a DH of 45% and hydrolysis conditions of 60°C temperature and a pH of 6.9, plotting E/S (%) against time shows a decreasing E/S ratio with an increasing hydrolysis time, as shown on figure 5.3. Non-linear regression analysis produced a logarithmic model with a high coefficient of determination (0.9996) (equation 16).

$$y = -1.799\,ln(x) + 4.6345 \qquad \textit{Equation 16}$$

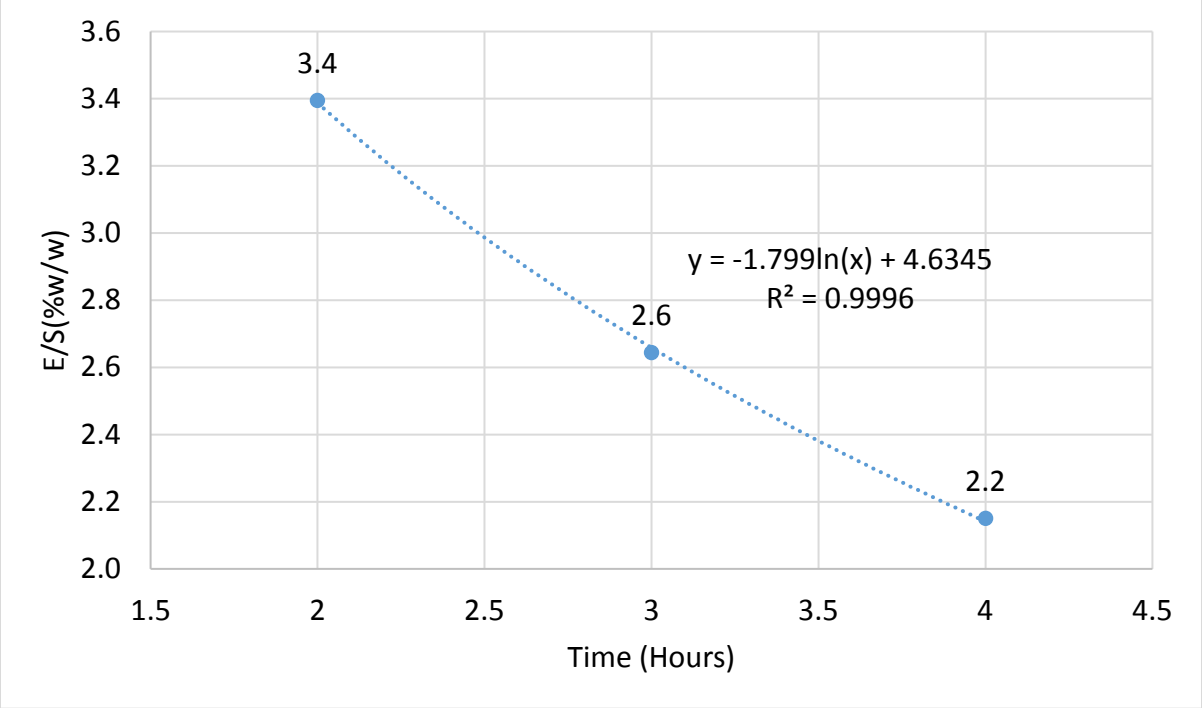

Figure 5-3; A graph of E/S (%) against time (minutes) at a constant DH of 45%

5.4 DISCUSSION

The increase in DH (Figure 5-1 and 5-2) with increasing E/S was also reported by other researchers (Bugg, 2004; Guerard *et al.*, 2001; Marangoni, 2003). When hydrolysing pacific whiting wastes with alcalase and neutrase, Benjakul and Morrissey (Benjakul & Morrissey, 1997) showed that increasing enzyme concentration resulted in higher degrees of hydrolysis. The results in this investigation are in agreement with those findings. The graph further shows that the E/S of between 1% and 2% could not achieve a DH of 45% in four hours of hydrolysis. The least E/S to achieve a DH of 45% was 3% (after 150 minutes of hydrolysis). Using E/S of 4% or 5% could quicken the rate of hydrolysis (attaining 45% DH within 90 minutes), but might not be cost effective, as enzymes are an important cost driver in enzymatic hydrolysis (Kim, 2011; Slizyte *et al.*, 2005). Figure 5-3 further supports these finding by showing a decline in the E/S ratio as hydrolysis time is increased to reach a constant DH of 45%. Thus to attain a similar DH, increasing hydrolysis time reduces the E/S ratio and vice versa. This graph, together with the resultant model (equation 16) can be used to predict the E/S ratio required to attain a DH of 45% at any given time. Its correlation of determination ($R^2 = 0.9996$) confirms its high reliability within the E/S ratio of 2-5% and a hydrolysis time of 2-4 hours, as per the experimental range.

The coefficients of determination are also very high for the linear relationships found between E/S and DH (0.96 -0.99) on Figure 5-2, confirming high data reliability. The linear relationship between E/S and DH was also reported by Guerard (Guerard *et al.*, 2001) who investigated the relationship between E/S and DH for tuna stomachs treated with alcalase, and found a linear relationship between the two characteristics, with strong correlation coefficients ($R = 0.99$). This further confirms the correspondence of the results of this investigation with that available in literature.

6. CONCLUSIONS

This research managed to establish the following conclusions;

i. The optimum operating conditions

Optimum operating conditions for the two enzymes, SEBPro XL and SEBDigest F59P on rainbow trout (*Oncorhynchus mykiss*) as the substrate were established. The two enzymes both operate optimally at a temperature of 60^{o}C. The optimum pH for SEBPro XL is 6.9 whilst that for SEBDigest F59P is 7.6. The enzyme concentrations at which the optimum conditions were established were 0.05% and 0.67% (w/w substrate concentration) for SEBDigest F59P and SEBPro XL respectively. Increasing E/S above these optimums will increase the DH, but the relative value per unit enzyme concentration will fall. This information will be a vital tool for the enzymatic hydrolysis industry, especially one that utilises these two enzymes and rainbow trout as the substrate.

ii. Combination treatments did not produce improved results

The enzyme SEBPro XL was found to be the best enzyme treatment from the five treatments that were evaluated for the hydrolysis of rainbow trout. It gave best results with regards to the DH, FAA concentration, and AA concentrations. Utilising enzyme combinations actually produced inferior results than the single enzyme SEBPro XL, especially in FAA, AA concentrations and DH. The exception was in CT3 which gave a higher content of micro minerals. This result will also be critical for the industry since it reduces enzyme costs on the process. It is also easier to handle one enzyme in a process than combinations.

iii. The macro and micro nutrient content

The resultant fish protein hydrolysate contains both micro and macro nutrients. Its heavy metal content is also lower than the limits gazetted by the regulatory authority, the Department of Agriculture, Forestry and fisheries. Therefore the FPH, besides being compliant to the environmental regulations, will also supply essential nutrients to the crops. The macro and micro nutrient contents were evidently influenced by the enzyme treatment applied, with enzyme treatment CT3 producing higher amounts of the nutrients than other enzyme treatments.

iv. Linear relationship between DH and E/S

By predetermining the reaction time and required DH, the E/S to be applied will be predicted. The DH can also be predicted from the reaction time and E/S applied. This result brings versatility to the hydrolysis process and will be welcomed by the industry. It eliminates uncertainty to the process since the graph can be used as reference tool to in decision making.

7. RECOMMENDATIONS

This work has shown that the rainbow trout FPH can optimally be produced at a set of hydrolysis conditions, chief among them reaction temperature, pH and enzyme to substrate (E/S) ratio. However, the following can still be improved for an improved product profile;

- Establish the product peptide molecular weight distribution

It was shown that one of the contributing factors the enzyme SEBPro XL was chosen for was the amount of free amino acids it liberated into the FPH. More free amino acids are favourable in the foliar fertiliser since plants can absorb them through their leaves and roots for various metabolic processes. Hydrolysis releases FAA and peptides of varying sizes, an FPH with more peptides of low molecular weight will be more favourable than the one with larger molecular weight. It will be vital to characterise the FPH's peptide molecular weight distribution as this will bring another way of distinguishing among the different FPH and aid in making a choice among them. Endo proteases do release less FAA than exoproteases, but might liberate a higher content of simpler peptides than exoproteases. This needs to be established.

- Field trials

Field trials should also be run to complement product analysis in providing information for both the producer and end user. The trials will help in establishing the absorption and effects of the product on plants.

- Establishing the product shelf life

The product shelf life should be established. Ways should be also found to stabilise the product, extend its shelf life and document the information.

- Economic evaluation

As a new product, the FPH's economic evaluation must be done to determine how much it shall cost to produce, sell and break even. This information will empower the producer in making decisions.

- Incorporating other enzymes

This work was undertaken with a view to combine, compare and select one of two pre-determined enzymes. The cooperation between these two enzymes did not produce a desirable, synergistic result, however combinations with other existing enzymes might produce a better outcome. SEBPro XL is an exoprotease, and an endoprotease compatible with it will add value to the hydrolysis. More experiments must be designed between SEBPro XL and other endoproteases in order to determine whether improved hydrolysis results are possible through synergistic effects between enzymes.

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9. APPENDIX

9.1 ENZYME SUPPLIER DATA

9.1.1 SEBDigest F59P Enzyme Data

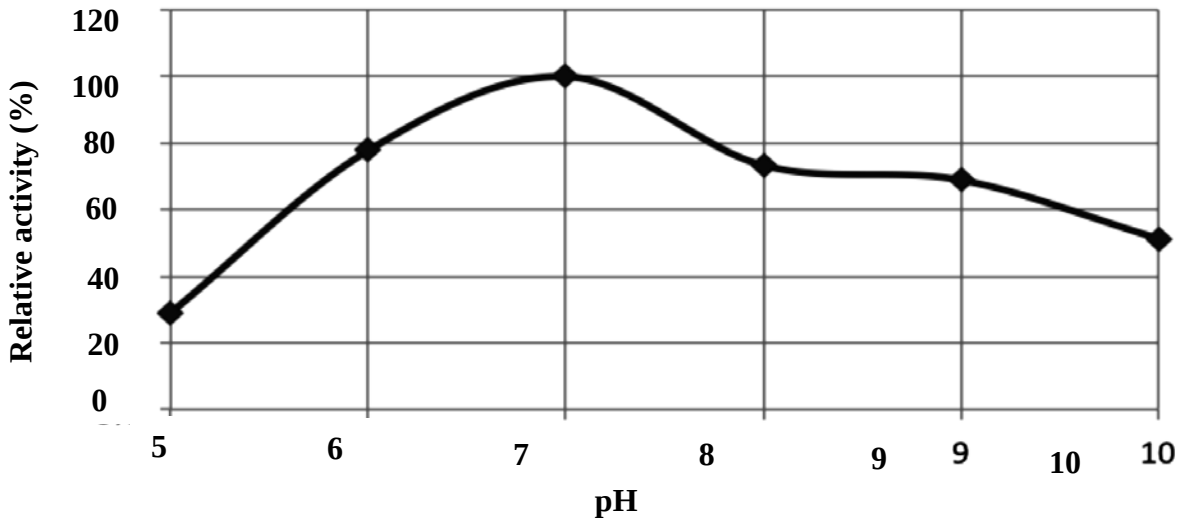

Figure 9-1; SEBDigest F59P pH profile at 37°C. Adapted from Advanced Enzymes (SEBDigest F59P) product data sheet

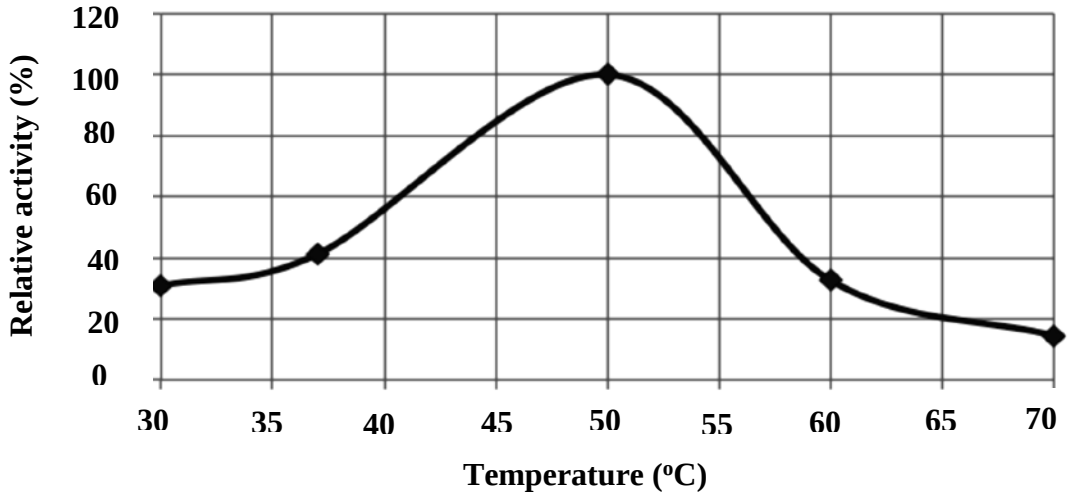

Figure 9-2; SEBDigest F59P temperature profile at pH 7. Adapted from Advanced Enzymes (SEBDigest F59P) product data sheet

9.1.2 SEBPro XL Enzyme Data

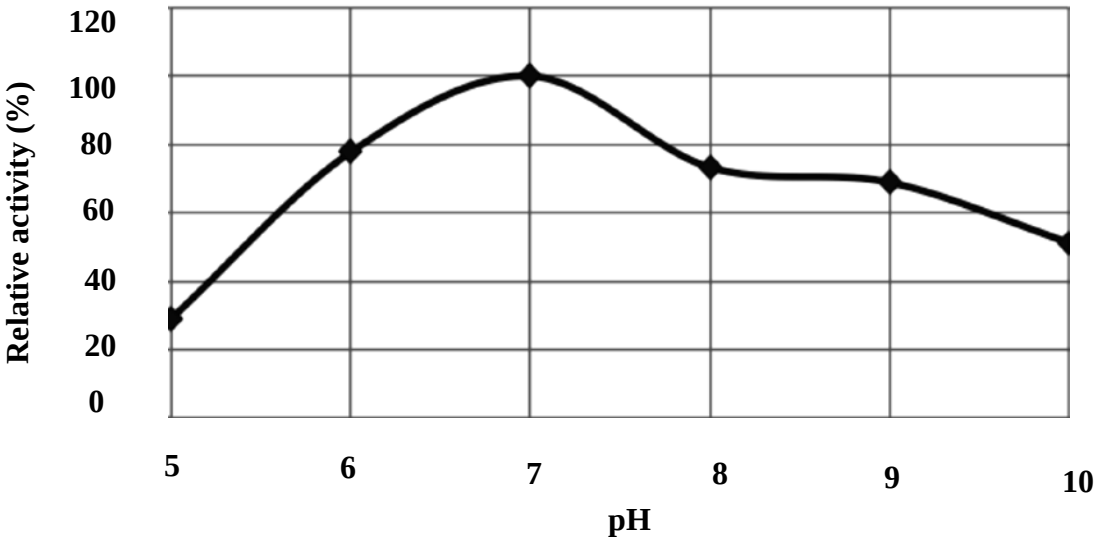

Figure 9-3; SEBPro XL pH profile at 40°C. Adapted from Advanced Enzymes, (SEBPro XL) product data sheet

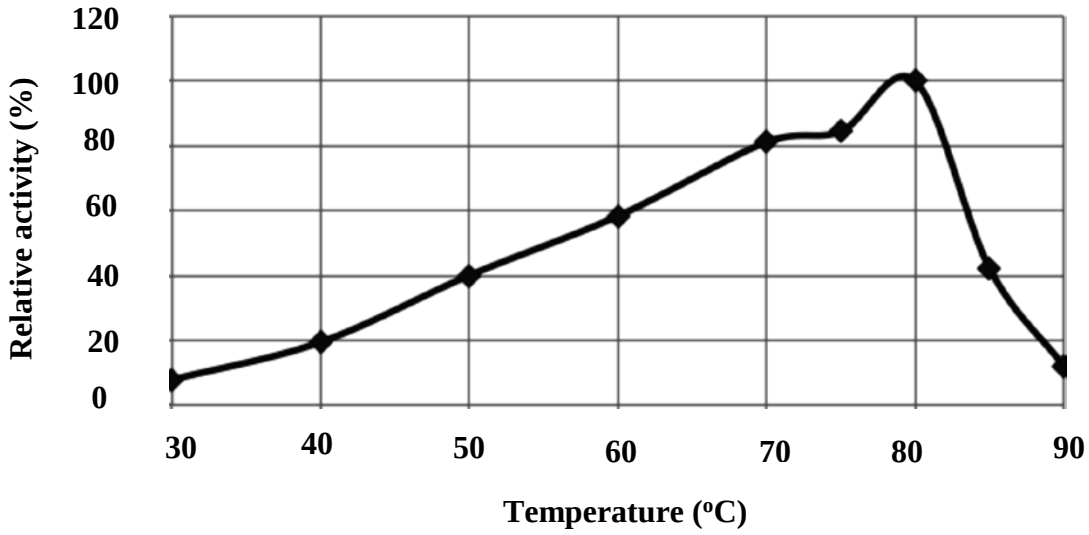

Figure 9-4; SEBPro XL temperature profile at pH 6. Adapted from Advanced Enzymes, (SEBPro XL) product data sheet

9.2 STATISTICS

9.2.1 Optimisation with SEBDigest F59P

Table 9-1; DH values at different factor levels, optimisation with SEBDigest F59P

Order number	E/S (%w/w)	Temperature ^{0}C	pH	DH %
1	0.15	55	10	4.3
2	0.05	55	8	17.4
3	0.2	40	9	11.0
4	0.2	40	7	10.6
5	0.1	40	9	10.2
6	0.25	55	8	9.2
7	0.15	55	6	7.2
8	0.2	70	7	7.4
9	0.1	70	9	9.6
10	0.15	30	8	10.5
11	0.1	40	7	11.4
12	0.1	70	7	11.7
13	0.2	70	9	6.7
14	0.15	80	8	7.7
15	0.15	55	8	13.2
16	0.15	55	8	12.6
17	0.15	55	8	12.7
18	0.15	55	8	13.0
19	0.15	55	8	12.9
20	0.15	55	8	13.5

Table 9-2; ANOVA showing DH as affected by E/S, pH and temperature, optimisation with SEBDigest F59P

	ANOVA; Var.:DH%; R-sqr=.96452; Adj:.93259 3 factors, 1 Blocks, 20 Runs; MS Res=.5956988 DV: DH%				
Factor	SS	df	MS	F	p
(1)E/S (L)	34.8083	1	34.80835	58.4328	0.000018
E/S (Q)	0.2387	1	0.23873	0.4008	0.540907
(2)Temp (L)	11.3962	1	11.39620	19.1308	0.001391
Temp (Q)	25.1345	1	25.13452	42.1933	0.000069
(3)pH (L)	5.6111	1	5.61112	9.4194	0.011859
pH (Q)	81.4413	1	81.44132	136.7156	0.000000
1L by 2L	6.4962	1	6.49621	10.9052	0.007982
1L by 3L	1.1757	1	1.17566	1.9736	0.190362
2L by 3L	0.4556	1	0.45564	0.7649	0.402321
Error	5.9570	10	0.59570		
Total SS	167.8914	19			

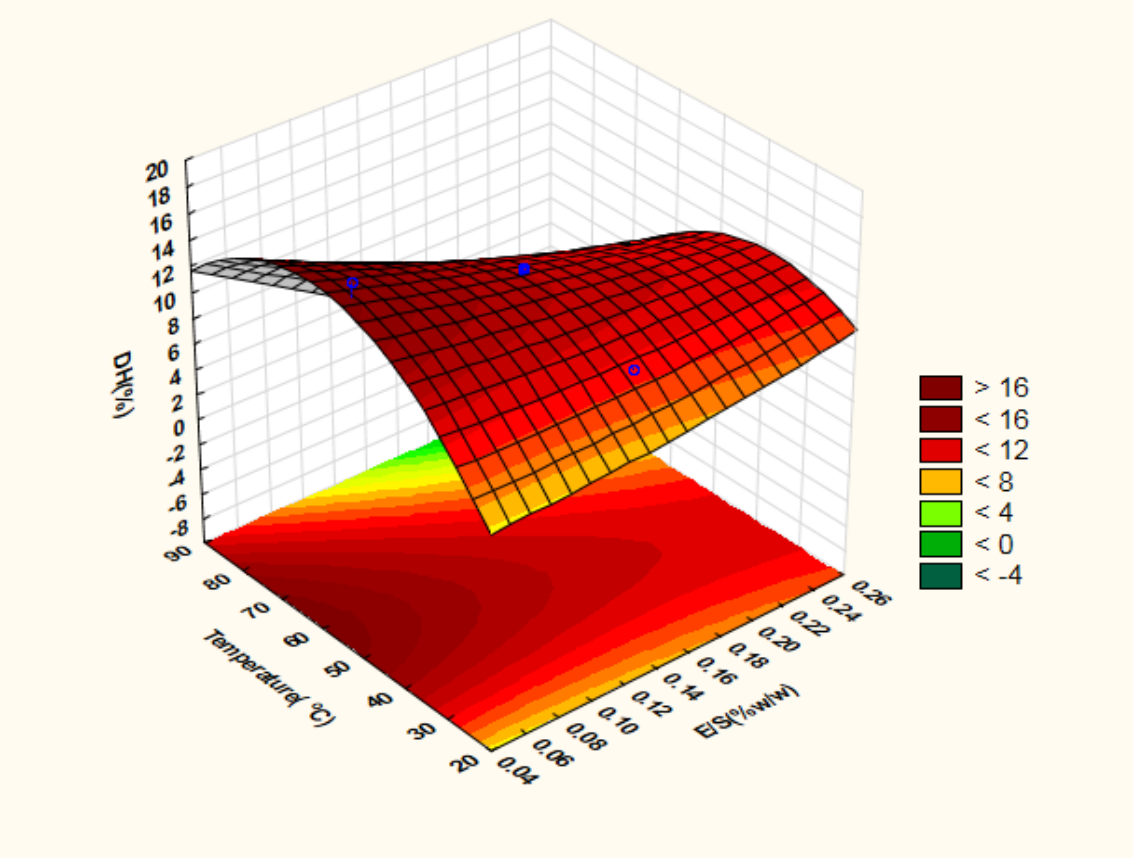

Figure 9-5; Response Surface showing the effects of temperature and E/S on DH, optimisation with SEBDigest F59P.

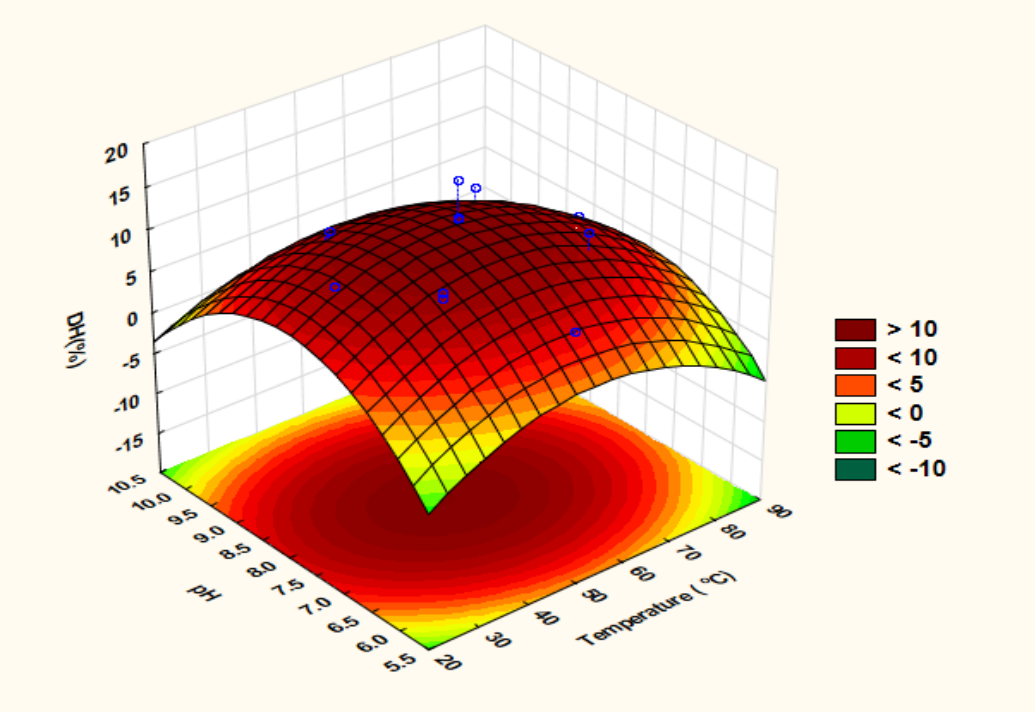

Figure 9-6; Response Surface showing DH as a function of temperature and pH, optimisation with SEBDigest F59P.

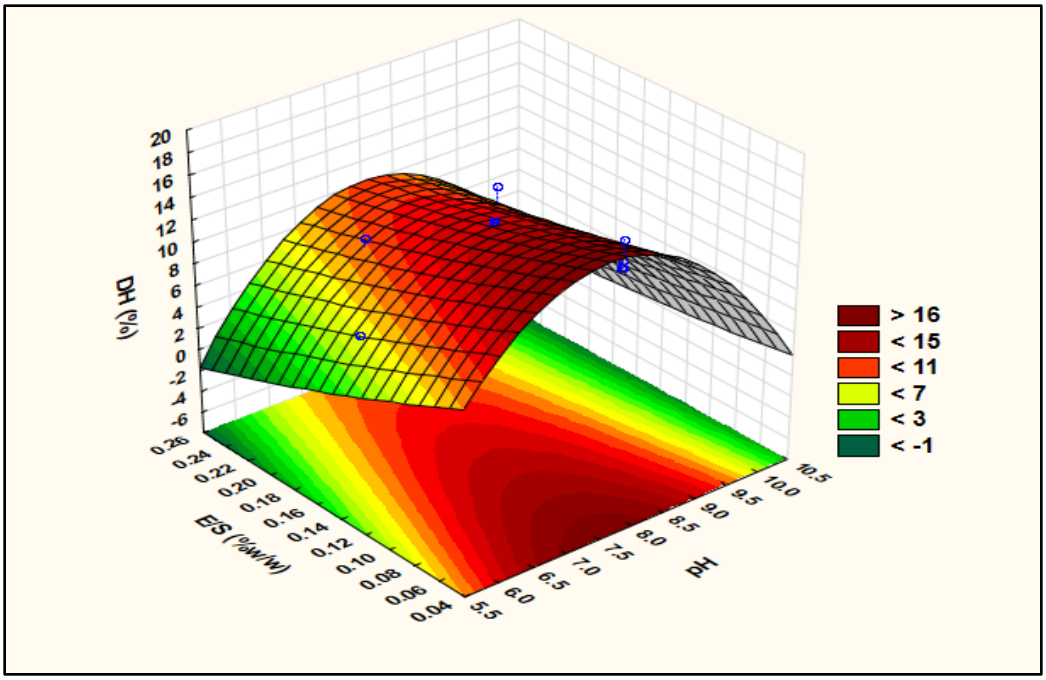

Figure 9-7; Response Surface showing DH as a function of E/S and pH, optimisation with SEBDigest F59P.

Table 9-3; Regression coefficients for the model equation, optimisation with SEBDigest F59P

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Factor	Regr. Coefficients; Var.:DH%; R-sqr=.96452; Adj:.93259 3 factors, 1 Blocks, 20 Runs; MS Residual=.5956988 DV: DH					
	Regressn Coeff.	Std.Err.	t(10)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	-114.036	15.37051	-7.4191	0.000023	-148.284	-79.7885
(1)E/S (L)	-36.352	51.54907	-0.7052	0.496785	-151.211	78.5062
E/S (Q)	38.665	61.07701	0.6330	0.540907	-97.423	174.7529
(2)Temp (L)	0.913	0.18677	4.8879	0.000634	0.497	1.3291
Temp (Q)	-0.006	0.00093	-6.4956	0.000069	-0.008	-0.0040
(3)pH (L)	27.699	2.77077	9.9967	0.000002	21.525	33.8723
pH (Q)	-1.785	0.15269	-11.6925	0.000000	-2.126	-1.4451
1L by 2L	-1.201	0.36384	-3.3023	0.007982	-2.012	-0.3908
1L by 3L	7.667	5.45756	1.4048	0.190362	-4.493	19.8272
2L by 3L	-0.016	0.01819	-0.8746	0.402321	-0.056	0.0246

Table 9-4; Validation run and predicted values, optimisation with SEBDigest F59P

pH	Temperature °C	E/S (%w/w)	DH (%)		
			Observed	Predicted	Residuals
0.1	45	8.5	12.0	12.9	0.9
0.2	40	7	10.9	10.1	-0.8
0.05	50	8	14.8	15.7	0.9
0.15	65	10	4.1	3.0	-1.1
0.1	70	7	11.9	12.6	0.7
0.2	55	9	10.8	9.5	-1.3
0.25	35	8	12.1	11.4	-0.7
0.05	50	10	5.4	6.0	0.6

9.2.2 Optimisation with SEBPro XL

Table 9-5; DH values at different factor levels, optimisation with SEBPro XL

Run Order	pH	Temperature (°C)	E/S (w/w)	DH %
1	6	40	0.4	16.6
2	6	40	1.0	15.2
3	6	70	0.4	19.9
4	6	70	1.0	13.9
5	8	40	0.4	12.6
6	8	40	1.0	15.8
7	8	70	0.4	15.7
8	8	70	1.0	14.9
9	5	55	0.7	17.4
10	9	55	0.7	15.4
11	7	30	0.7	17.7
12	7	80	0.7	20.5
13	7	55	0.2	11.8
14	7	55	1.2	10.9
15	7	55	0.7	23.1
16	7	55	0.7	22.8
17	7	55	0.7	22.3
18	7	55	0.7	23.0

Table 9-6; ANOVA showing DH as affected by E/S, pH and temperature, optimisation with SEBPro XL.

Factor	ANOVA; Var.:DH%; R-sqr=.99301; Adj:.98515 (2**(3) 3 factors, 1 Blocks, 18 Runs; MS Residual=.2291187 DV: DH				
	SS	df	MS	F	p
(1)pH (L)	7.2691	1	7.2691	31.7263	0.000491
pH (Q)	59.9715	1	59.9715	261.7485	0.000000
(2)Temp (L)	5.8118	1	5.8118	25.3658	0.001006
Temp (Q)	18.9142	1	18.9142	82.5521	0.000017
(3)E/S(L)	3.1067	1	3.1067	13.5592	0.006199
E/S(Q)	198.6459	1	198.6459	866.9997	0.000000
1L by 2L	0.0050	1	0.0050	0.0218	0.886215
1L by 3L	12.0050	1	12.0050	52.3964	0.000089
2L by 3L	9.2450	1	9.2450	40.3503	0.000220
Error	1.8329	8	0.2291		
Total SS	262.2894	17			

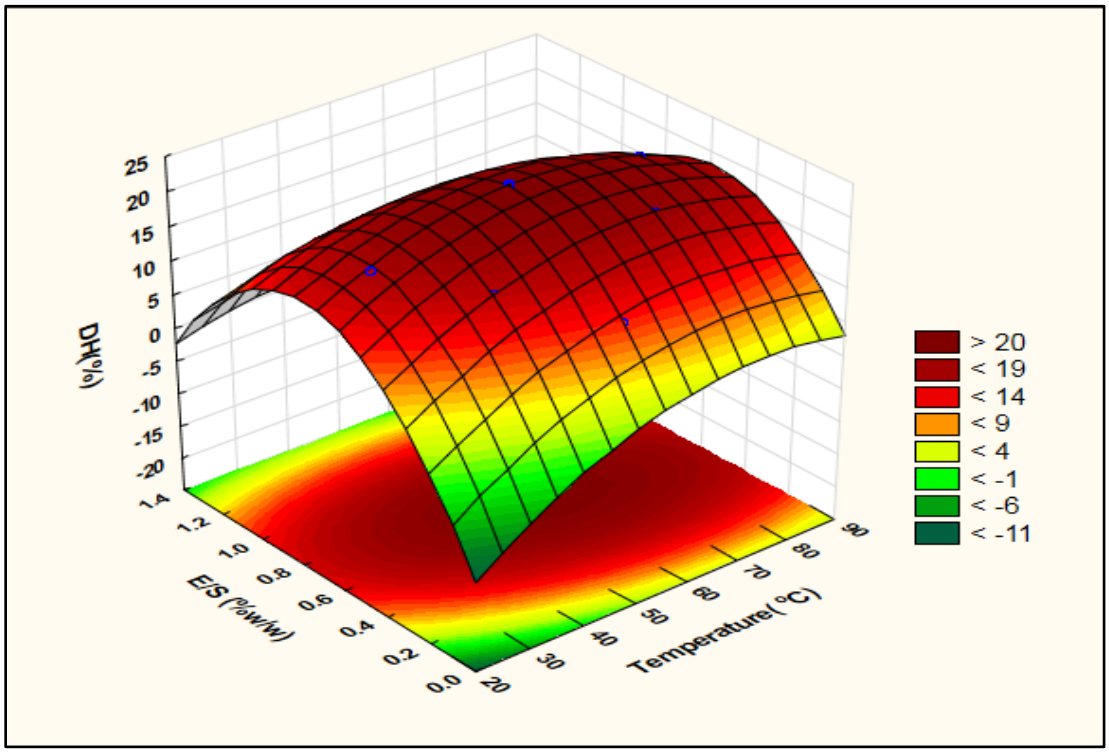

Figure 9-8; Response Surface showing the effects of temperature and E/S on DH, optimisation with SEBPro XL.

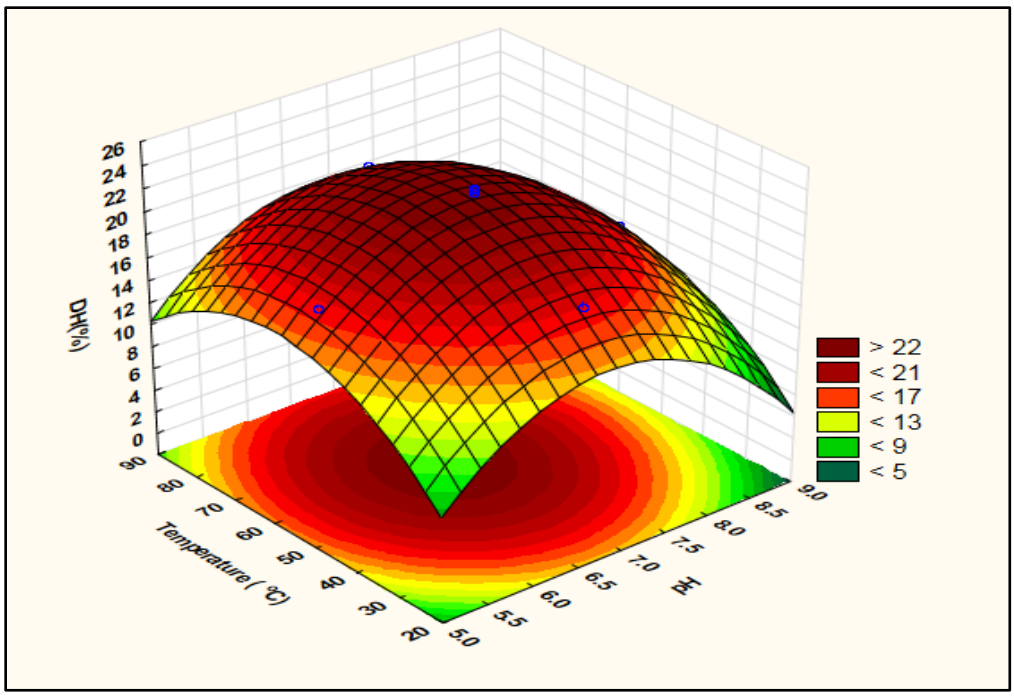

Figure 9-9; Response Surface showing the effects of temperature and pH on DH, optimisation with SEBPro XL.

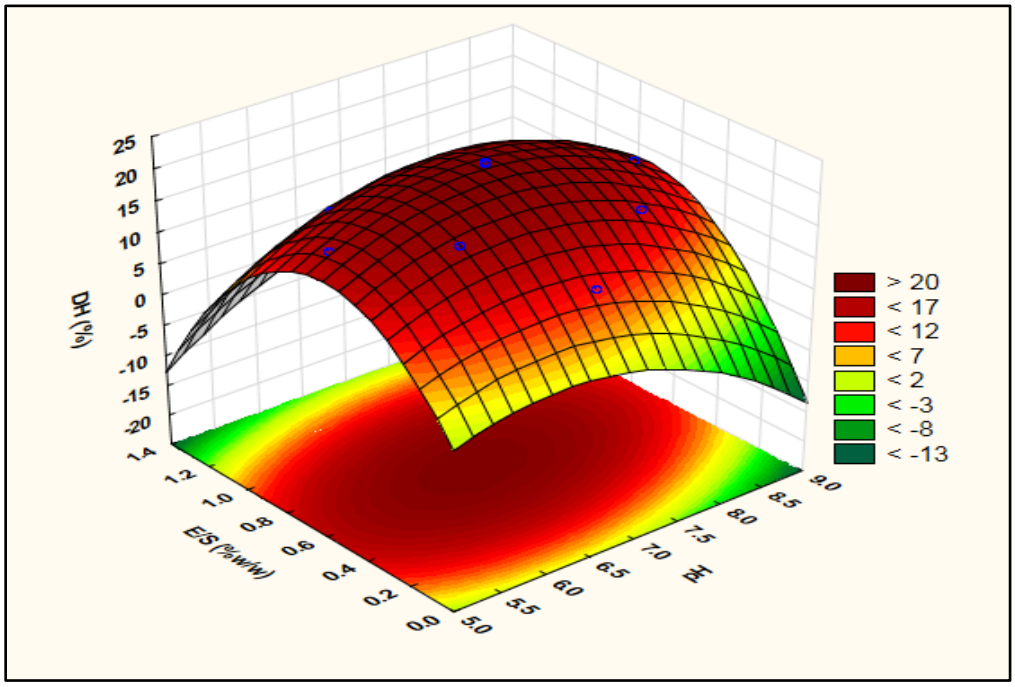

Figure 9-10; Response Surface showing the effects of pH and E/S on DH, optimisation with SEBPro XL.

Table 9-7; Regression coefficients for the model equation, optimisation with SEBPro XL

Factor	Regr. Coefficients; Var.:DH%; R-sqr=.99301; Adj:.98515 (2**(3) 3 factors, 1 Blocks, 18 Runs; MS Residual=.2291187 DV: DH					
	Regressn Coeff.	Std.Err.	t(8)	p	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	-106.648	9.057106	-11.7750	0.000002	-127.533	-85.7618
(1)pH (L)	26.804	2.026812	13.2248	0.000001	22.130	31.4781
pH (Q)	-2.177	0.134586	-16.1786	0.000000	-2.488	-1.8671
(2)Temp (L)	0.797	0.106461	7.4851	0.000070	0.551	1.0424
Temp (Q)	-0.005	0.000598	-9.0858	0.000017	-0.007	-0.0041
(3)E/S(L)	44.610	4.943733	9.0236	0.000018	33.210	56.0105
E/S(Q)	-44.032	1.495398	-29.4449	0.000000	-47.480	-40.5834
1L by 2L	0.002	0.011282	0.1477	0.886215	-0.024	0.0277
1L by 3L	4.083	0.564110	7.2385	0.000089	2.782	5.3842
2L by 3L	-0.239	0.037607	-6.3522	0.000220	-0.326	-0.1522

Table 9-8; Validation run and predicted values, optimisation with SEBPro XL

pH	Temperature °C	E/S (%w/w)	DH (%)		
			Observed	Predicted	Residuals
6	40	0.4	16.1	16.9	0.8
8	55	1.2	13	11.6	-1.4
6	40	0.9	19.3	18.1	-1.2
9	55	0.5	10.4	11.0	0.6
7	55	0.7	25.1	24.2	-0.9
6	70	0.6	22.3	23.5	1.2
6	40	0.8	18.9	19.6	0.7
7	80	0.4	23	21.8	-1.2

9.3 DH PROFILING

Table 9-9; DH values of hydrolysis reactions with the five enzyme treatments

	DH%				
Time (mins)	SEBPro XL	SEBDigest F59P	CT1	CT2	CT3
30	14.5	11.2	14.8	10.9	20.1
60	21.1	17.6	23.1	18.1	27.0
90	29.0	21.1	29.6	20.1	30.1
120	31.5	24.1	31.3	23.3	31.9
150	34.0	25.2	33.0	29.8	32.5
180	35.0	26.1	33.7	31.1	34.4

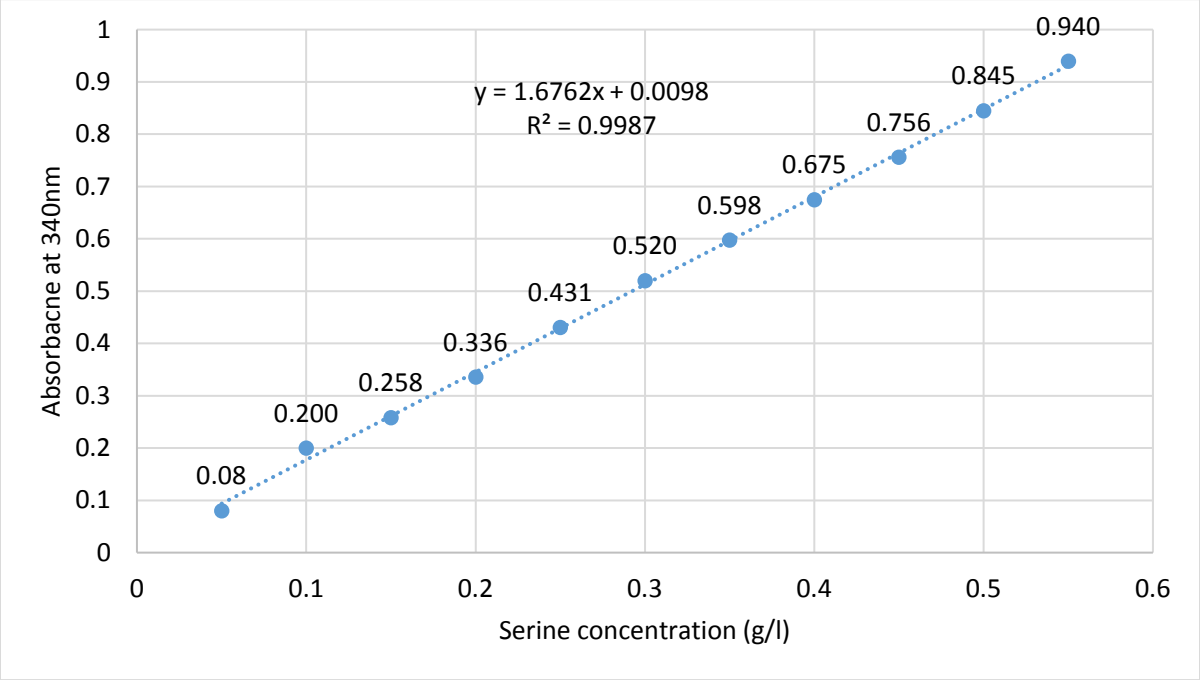

Figure 9-11; The serine standard curve for the OPA method of DH calculation

9.4 MINERAL PROFILING

Table 9-10; mineral profiles for the five FPH after 3 hours of hydrolysis

Element	Concentration (ppm)				
	SEBPro XL	SEBDigest 59P	CT2	CT1	CT3
Li	< 3	< 3	< 3	< 3	< 3
Be	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
B	20012.5	13998.1	11163.4	18606.0	13424.9
Al	13045.2	10896.6	9327.2	9277.0	9774.3
Cr	54.9	55.3	55.7	58.8	58.9
Mn	1458.7	595.1	785.2	1024.1	6242.6
Fe	32909.6	25201.9	27358.0	27143.2	50764.4
Co	121.7	93.6	84.8	85.9	155.0
Ni	37.5	28.4	27.5	29.2	95.4
Cu	4156.5	2661.1	1584.1	1853.7	2914.6
Zn	36578.4	25136.0	27771.4	28341.4	70209.0
As	1722.6	1708.9	1586.7	1696.9	1714.3
Se	200.5	169.9	132.7	175.3	248.3
Mo	62.0	12.0	5.6	6.3	22.4
Cd	16.9	10.6	14.1	11.8	23.5
Hg	5.9	6.1	2.7	5.6	5.9
Pb	26.9	22.7	26.8	35.6	35.0
Ba	8.3	6.8	5.6	6.0	6.8
Ca	2604.0	1337.4	1879.2	1864.2	17058.0
K	9536.0	10602.0	11826.0	11134.0	15068.0
Mg	160.0	90.0	107.1	115.2	694.8
Na	1902.8	1403.6	1443.0	1404.6	2494.0
P	54980.0	42300.0	41740.0	43400.0	60360.0
Si	33.8	23.7	22.7	28.3	26.1
Sr	4.4	1.9	3.0	3.2	25.8

9.5 AMINO ACID PROFILING

Table 9-11; Total AA composition in FPH of the five enzyme treatments after 3 hour of hydrolysis

	Enzyme Treatment				
Amino acid (ppm)	SEBPro XL	SEBDigest F59P	CT1	CT2	CT3
Histidine	6796.2	1423.8	1281.0	1044.9	1513.2
Serine	12206.1	2309.4	2281.2	2029.2	3576.0
Arginine	24139.5	4156.5	4279.8	3887.4	6285.0
Glycine	37464.0	6572.1	7060.5	6463.5	10739.7
Aspartic acid	14988.3	3600.0	3525.3	2994	6244.5
Glutamic acid	25497.0	5822.1	5941.5	4822.5	9926.7
Threonine	9756.6	1246.2	1874.7	1508.4	2759.4
Alanine	14566.2	2947.2	2979.3	2429.4	5182.8
Proline	16824.6	2937.9	2962.5	2450.7	4818.0
Lysine	6498.0	1359.3	1637.1	1085.1	2523.6
Tyrosine	9018.9	1452.0	1159.5	1110.0	1669.2
Methionine	7001.7	0	0	0	315.6
Tryptophan	182.0	85.1	63.9	81.7	99.6
Valine	10344.9	1949.1	1740.9	1387.8	2725.8
Isoleucine	5788.2	532.8	1039.8	774.0	1431.9
Leucine	15201.0	3534.6	3095.1	2321.1	4551.9
Phenylalanine	14005.5	2526.3	2496.6	2055.9	3107.4
Asparagine	8032.2	868.8	952.2	923.7	1171.8
OH Proline	10457.4	2199.0	1926.3	1835.1	3018.6

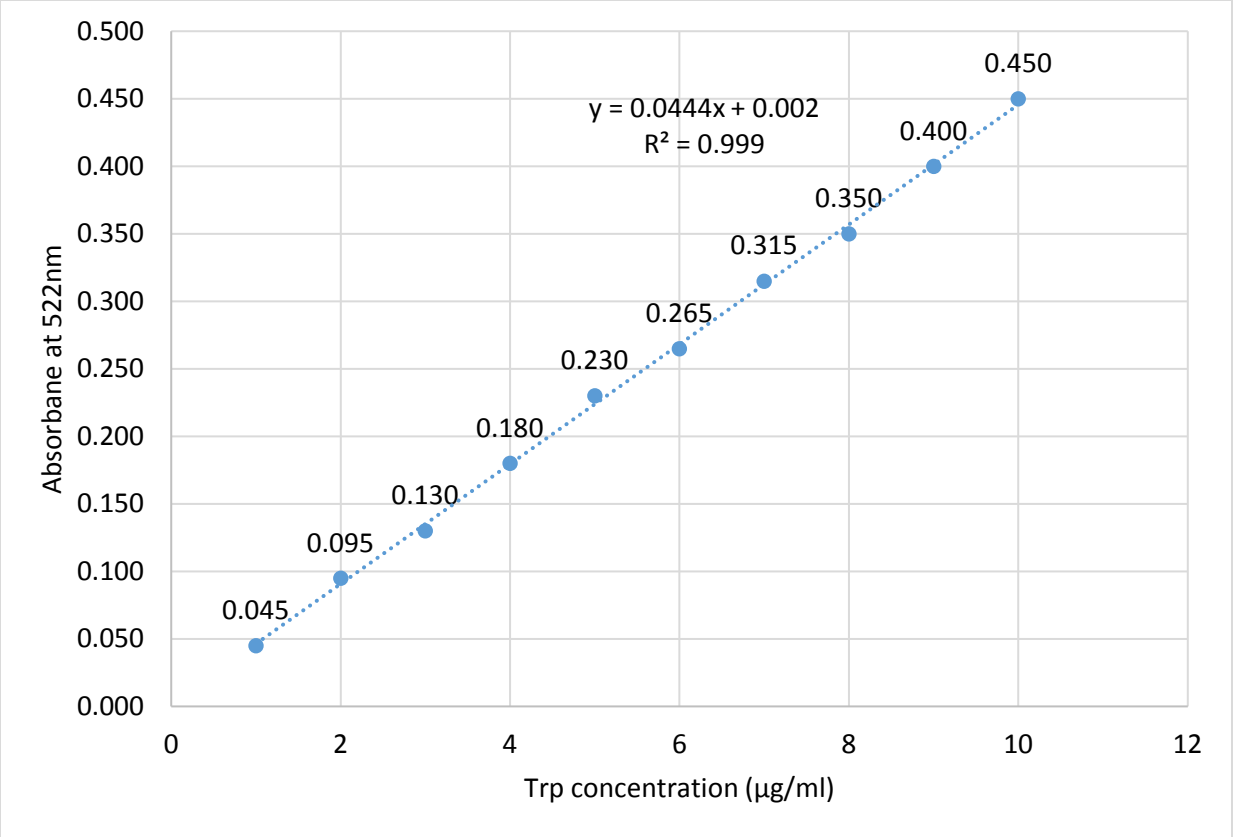

Figure 9-12; The tryptophan standard curve

9.6 FREE AMINO ACID PROFILING

Table 9-12; A table showing total FAA in FPH of the five enzyme treatments

	Total free AA (ppm)				
Time (minutes)	SEBPro XL	SEBDigest F59P	CT1	CT2	CT3
30	15079.0	11394.2	15143.3	10414.9	10314.9
60	18494.2	11723.8	18039.8	11559.1	10100.6
90	26712.6	12793.8	26298.7	12642.0	13751.6
120	35259.9	12784.2	13685.8	12604.3	16587.5
150	38105.1	14939.3	12116.6	9971.4	17493.7
180	30429.4	16531.9	18572.3	11089.4	21093.1

Table 9-13; FAA compositions in the FPH catalysed by SEBPro XL with time

Free AA (ppm)	Time (minutes)					
	30	60	90	120	150	180
Histidine	446.4	531.8	731.6	809.6	876.7	618.4
Serine	759.4	844.6	1222.7	1589.4	1600.4	1305.9
Arginine	1431.3	2031.4	3082.4	4612.3	4235.8	3386.3
Glycine	943.2	1440.6	2111.5	3229.2	3303.7	2412.7
Aspartic acid	1198.7	1340.6	1800.0	2581.8	2406.9	1936.3
Glutamic acid	1386.2	1564.9	2027.4	2598.4	2650.5	2255.8
Threonine	619.0	681.0	942.4	1462.3	1265.2	1106.6
Alanine	1273.5	1504.5	2188.0	2894.4	3152.2	2581.8
Proline	914.2	1353.1	1714.0	2284.3	2496.7	1808.3
Lysine	900.5	1079.7	1362.1	1689.7	2234.4	2150.2
Tyrosine	639.3	964.7	1505.3	1746.5	2294.4	1685.5
Methionine	326.1	262.1	441.8	708.1	914.1	730.5
Valine	722.7	994.3	1440.0	1768.6	2121.9	1718.1
Isoleucine	539.2	680.3	1009.9	1358.4	1501.1	1204.1
Leucine	1122.6	1569.6	2308.8	2879.6	3712.3	2954.4
Phenylalanine	1496.0	1256.1	2379.7	2436.5	2594.8	1972.7
Asparagine	135.4	103.4	89.9	113.4	120.3	93.1
Glutamine	225.2	291.6	355.0	497.6	623.8	508.7

Table 9-14; FAA variations in the FPH catalysed by SEBDigest F59P with time

Free AA (ppm)	Time (Minutes)					
	30	60	90	120	150	180
Histidine	324.8	255.0	219.9	156.9	171.2	150.6
Serine	546.8	496.2	557.9	534.5	674.2	706.3
Arginine	768.2	837.8	1034.2	1019.6	1188.6	1346.5
Glycine	1016.5	1125.2	1112.2	1143.2	1321.1	1565.4
Aspartic acid	995.9	951.1	1090.7	1032.9	1207.0	1318.8
Glutamic acid	1118.6	1016.9	1149.6	1090.7	1281.7	1408.5
Threonine	438.4	415.0	468.6	449.8	570.1	604.0
Alanine	1016.7	993.9	1088.5	1075.3	1234.2	1418.8
Proline	1140.6	1124.5	937.9	1019.8	1298.7	1349.4
Lysine	761.9	835.2	1007.2	1078.5	930.6	1187.7
Tyrosine	526.3	583.5	609.1	595.4	669.0	826.3
Methionine	174.5	202.3	155.4	183.0	156.2	167.4
Valine	569.4	585.2	732.9	709.1	845.6	947.7
Isoleucine	417.6	435.3	470.9	469.2	544.0	646.2
Leucine	917.0	1009.0	1019.5	1026.6	1139.4	1412.2
Phenylalanine	530.8	679.8	1003.6	935.4	1590.1	1247.9
Asparagine	57.6	98.1	102.4	113.0	65.4	152.8
Glutamine	72.6	79.7	33.1	151.4	52.3	75.5

Table 9-15; FAA variations in the FPH catalysed by CT1 with time

Free AA (ppm)	Time (minutes)					
	30	60	90	120	150	180
Histidine	442.0	537.2	696.1	348.5	317.5	439.1
Serine	752.0	853.1	1352.6	692.5	571.9	889.7
Arginine	1542.2	2051.9	2932.7	1965.2	1697.3	2533.9
Glycine	934.0	1455.2	2009.0	745.4	755.1	1011.8
Aspartic acid	1256.8	986.0	1712.6	954.9	820.6	1313.6
Glutamic acid	1372.6	1580.7	2400.5	1248.3	1069.4	1602.2
Threonine	612.9	687.9	100.9	547.9	474.1	720.8
Alanine	1261.0	1519.7	2081.8	1159.9	986.3	1647.7
Proline	905.2	1200.0	1630.8	631.9	697.6	854.4
Lysine	891.7	1090.7	1498.3	1076.9	594.5	1047.2
Tyrosine	633.0	974.5	1652.1	597.7	583.7	726.3
Methionine	322.9	264.7	420.4	27.6	36.9	9.3
Valine	715.6	898.0	1370.1	771.4	672.0	1080.8
Isoleucine	551.3	687.1	1178.2	521.3	484.0	763.6
Leucine	1111.6	1585.5	2456.5	1131.3	1055.4	1511.9
Phenylalanine	1481.3	1268.7	2264.2	989.2	1071.8	2292.9
Asparagine	134.1	104.4	85.6	117.7	108.6	127.2
Glutamine	223.0	294.5	456.3	158.2	119.9	

Table 9-16; FAA variations in the FPH catalysed by CT2 with time

Free AA (ppm)	Time (minutes)					
	30	60	90	120	150	180
Histidine	282.8	294.3	317.0	270.5	237.6	242.9
Serine	508.4	680.1	771.4	744.5	588.4	636.9
Arginine	734.1	927.7	1088.8	1147.3	870.5	1026.5
Glycine	771.1	741.5	815.7	853.9	648.2	717.5
Aspartic acid	844.5	1095.0	1210.2	1195.4	913.7	1044.2
Glutamic acid	1004.5	1249.1	1461.8	1450.4	1151.8	1253.2
Threonine	419.9	522.6	601.4	581.6	470.5	510.4
Alanine	928.1	1105.6	1179.4	1157.4	914.9	1091.7
Proline	873.1	699.3	764.5	799.9	624.4	627.7
Lysine	284.2	732.9	838.8	675.0	520.6	594.2
Tyrosine	393.7	451.6	492.8	484.1	351.5	401.7
Valine	560.2	651.7	700.7	724.3	577.7	619.0
Isoleucine	362.1	432.5	471.6	487.0	370.6	398.2
Leucine	750.0	910.5	997.9	982.5	764.1	821.9
Phenylalanine	1484.7	986.5	878.0	861.1	748.2	1007.7
Asparagine	137.7	54.0		56.5	77.6	34.3
Glutamine	75.6	24.2		122.7	92.9	29.9

Table 9-17; FAA variations in the FPH catalysed by CT3 with time

Free AA (ppm)	Time (minutes)					
	30	60	90	120	150	180
Histidine	224.2	165.2	149.4	243.3	227.8	220.2
Serine	485.9	465.3	637.7	939.6	788.5	1047.8
Arginine	927.1	1004.1	1297.0	1749.1	1675.6	1633.1
Glycine	731.2	843.3	912.1	1111.3	1583.4	1866.3
Aspartic acid	849.0	745.3	1047.9	1328.4	1239.8	1498.3
Glutamic acid	911.0	845.0	1192.0	1593.9	1433.3	1673.4
Threonine	373.8	373.3	534.1	711.8	641.9	816.4
Alanine	854.9	852.6	1149.4	1523.4	1567.1	2026.9
Proline	727.4	788.1	815.8	973.1	1462.6	1689.9
Lysine	322.5	322.9	552.5	838.7	811.6	882.5
Tyrosine	452.0	455.3	568.4	688.2	911.2	933.1
Methionine	14.4	42.8	59.0	64.8	93.6	84.8
Valine	541.2	563.4	813.6	975.0	1010.1	1254.4
Isoleucine	357.3	381.7	461.2	648.3	684.6	830.3
Leucine	726.6	809.2	963.5	1297.6	1604.8	1862.2
Phenylalanine	1735.1	1254.6	2340.9	1776.5	1450.8	2397.9
Asparagine	81.4	85.8	129.9	124.5	153.8	177.8
Glutamine		102.7	127.2		153.2	197.8

9.7 CORRELATION ANALYSIS

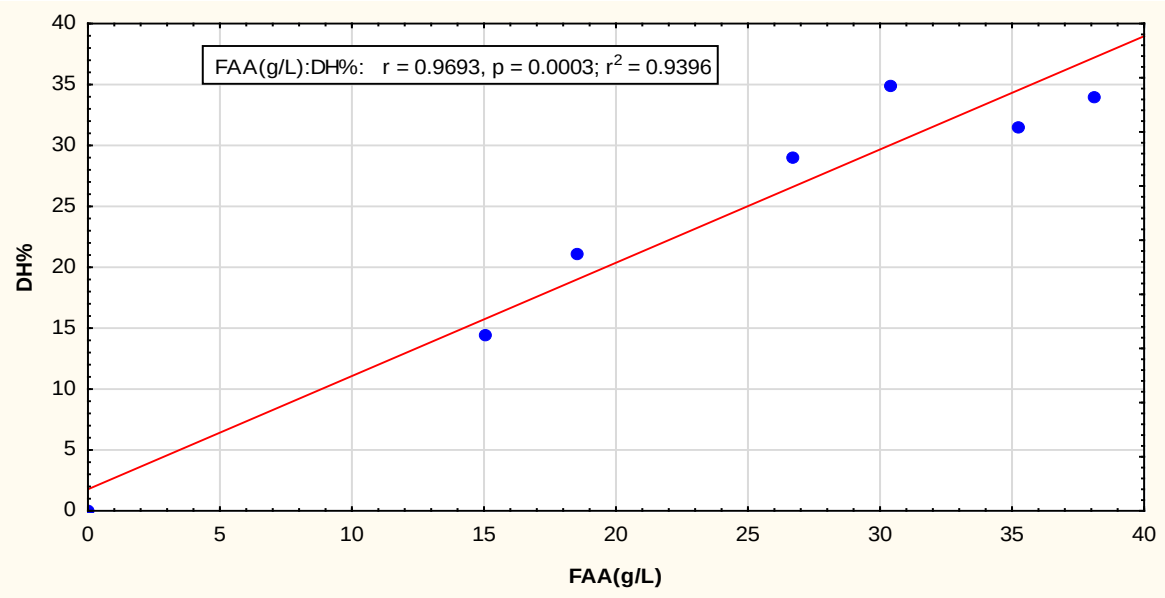

Figure 9-13; DH% against FAA (g/L) for a reaction catalysed by SEBPro XL

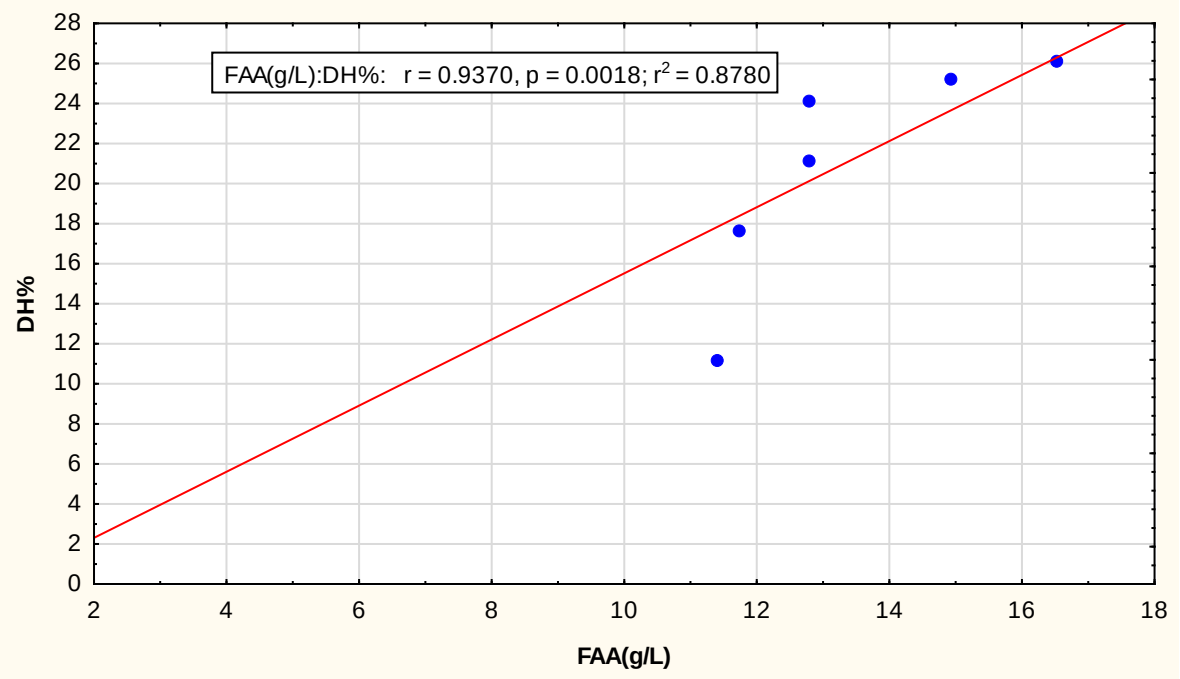

Figure 9-14; DH% against FAA (g/L) for a reaction catalysed by SEBDigest F59P

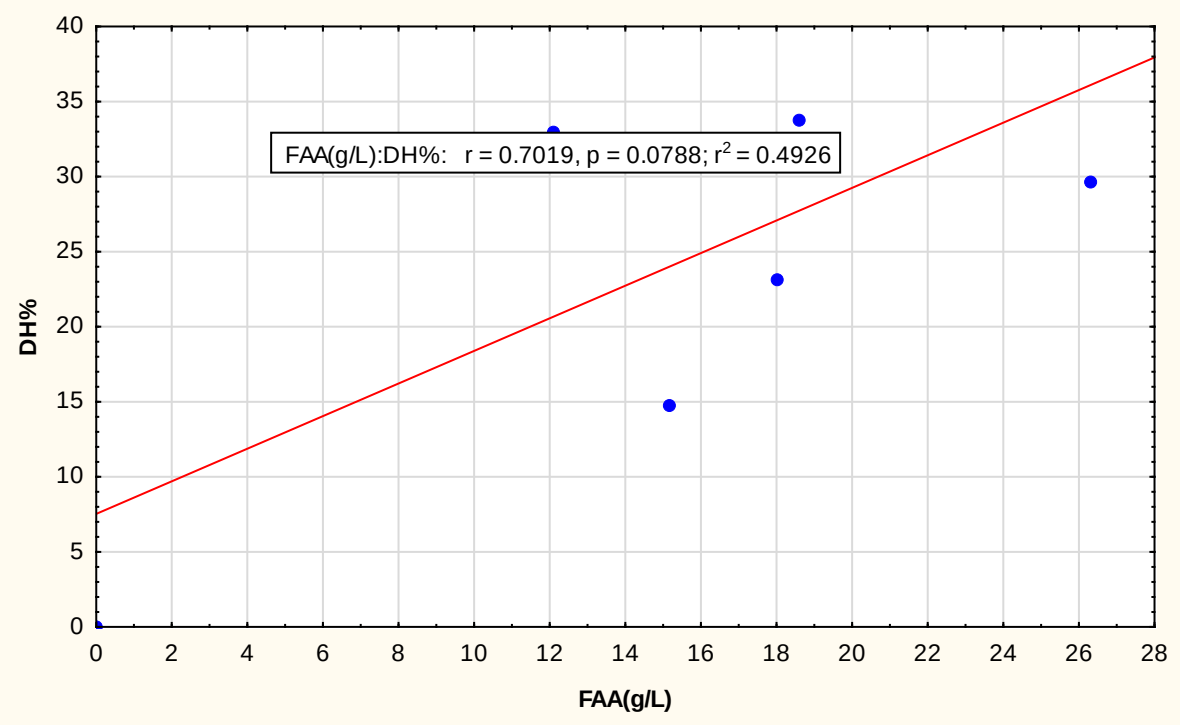

Figure 9-15; DH% against FAA (g/L) for a reaction catalysed by CT1

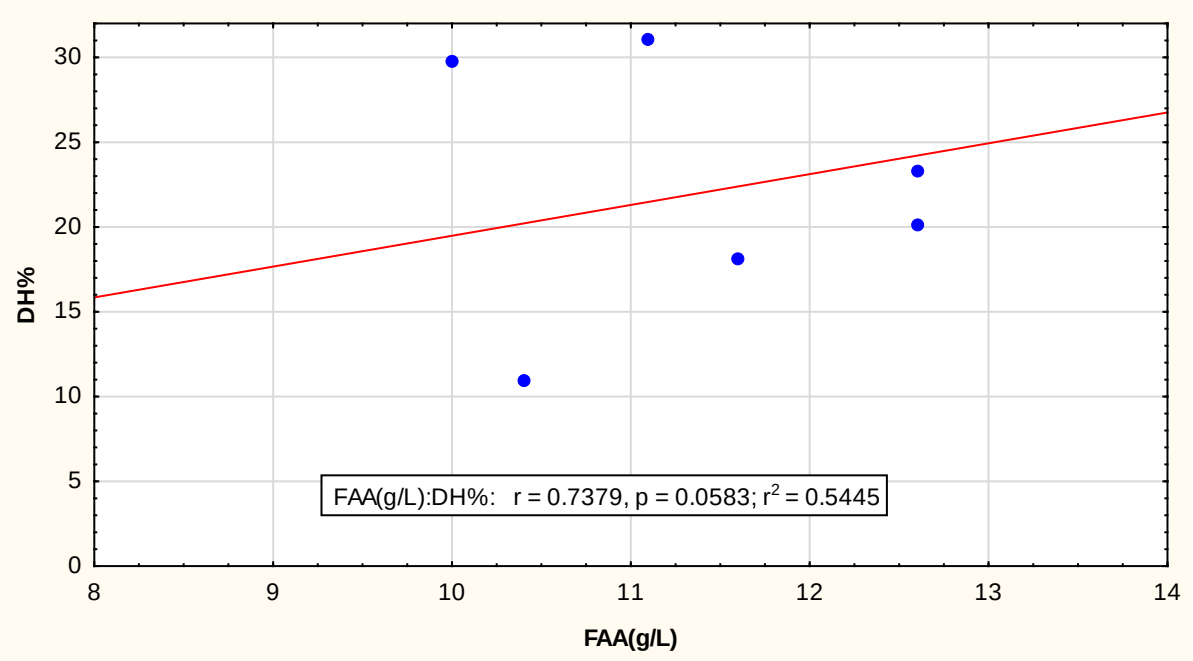

Figure 9-16; DH% against FAA (g/L) for a reaction catalysed by CT2

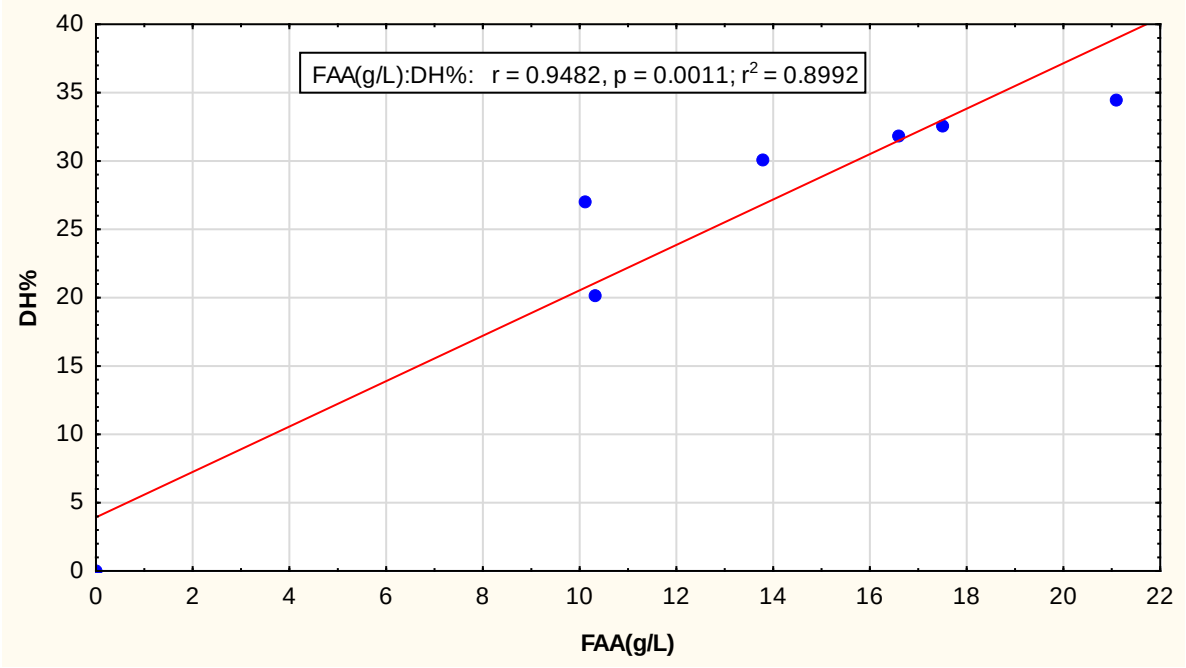

Figure 9-17; DH% against FAA (g/L) for a reaction catalysed by CT3

9.8 DH FOR 1-5% E/S

Table 9-18; Variation of DH with time for varying E/S when catalysing with SEBPro XL (1-5% E/S)

	DH%				
Time(mins)	E/S=5%	E/S=4%	E/S=3%	E/S=2%	E/S=1%
0	0.0	0.0	0.0	0.0	0.0
30	30.7	25.2	23.5	19.2	14.9
60	39.1	36.8	34.6	29.8	24.6
90	47.5	46.1	41.7	34.6	26.3
120	53.7	49.2	43.0	37.2	30.2
150	58.8	53.4	45.8	38.3	33.6
180	59.5	55.6	47.5	39.1	36.2
210	60.24	56.8	49.1	42.3	37.8
240	60.02	58.2	50.9	43.1	38.0

9.9 PROGRESS CURVES OF FREE AMINO ACIDS FOR THE FIVE DIFFERENT FPH

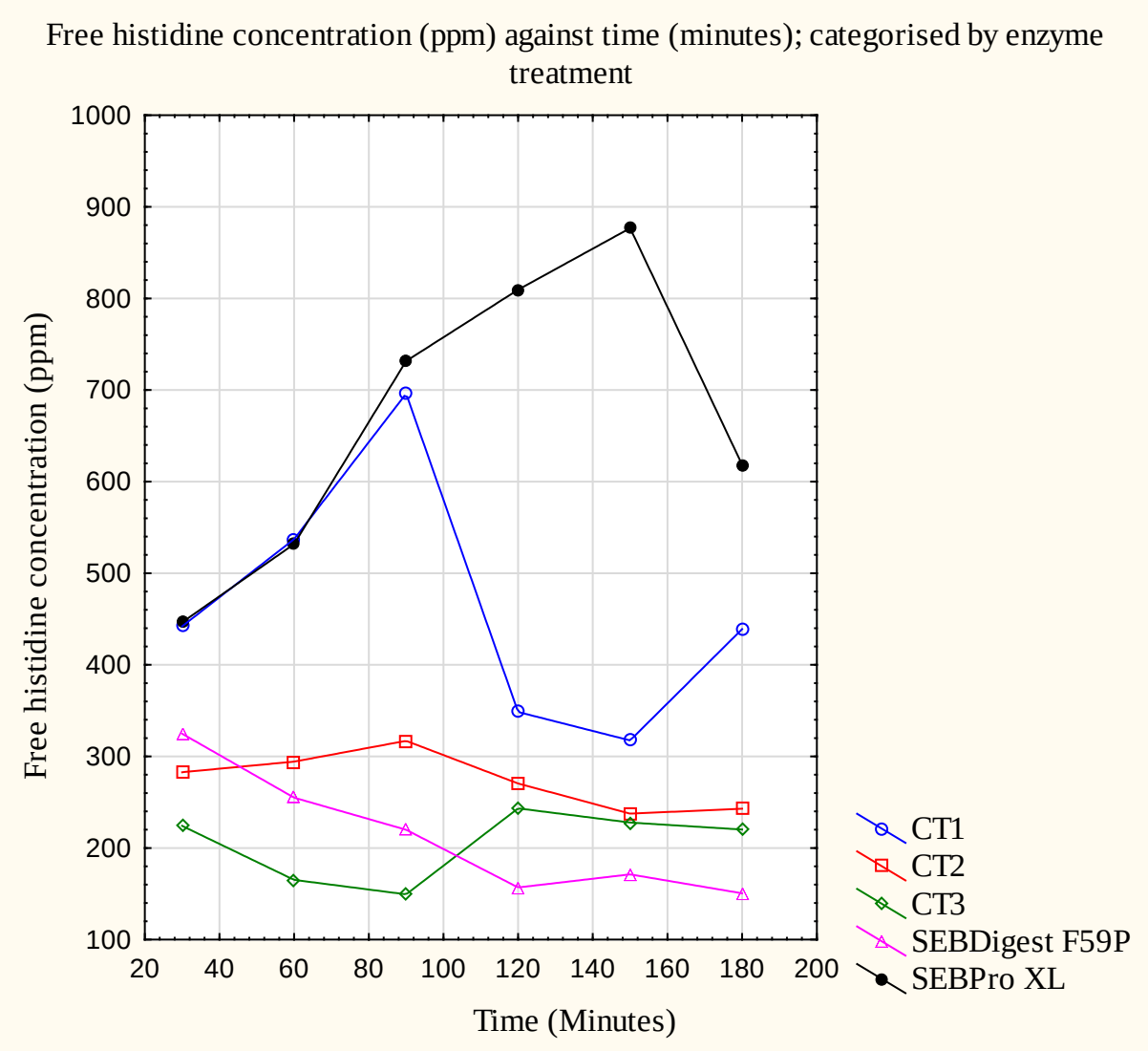

Figure 9-18; Progress curves for free histidine concentration with time for the five different enzyme treatments

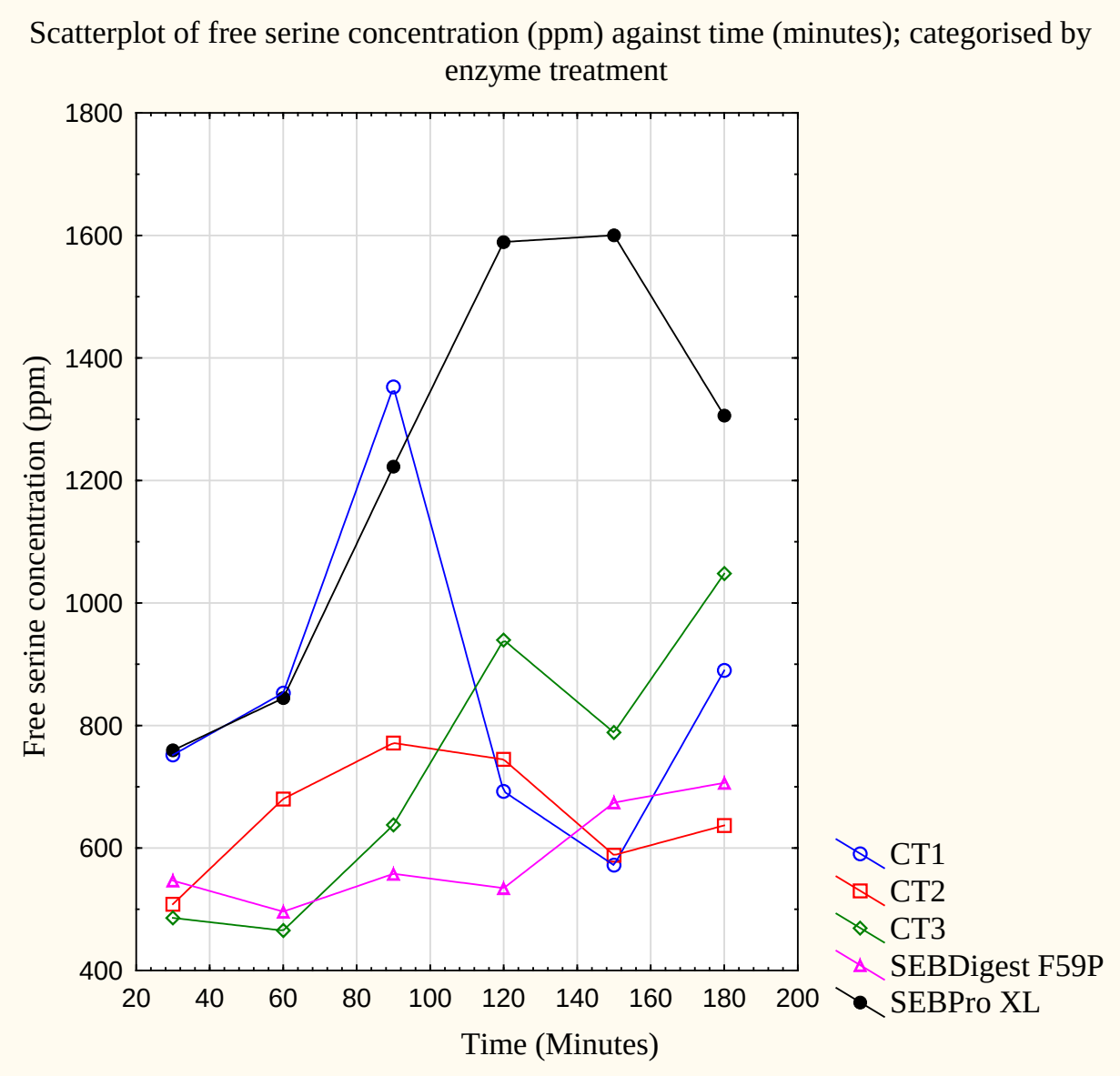

Figure 9-19; Progress curves for free serine concentration with time for the five different enzyme treatments

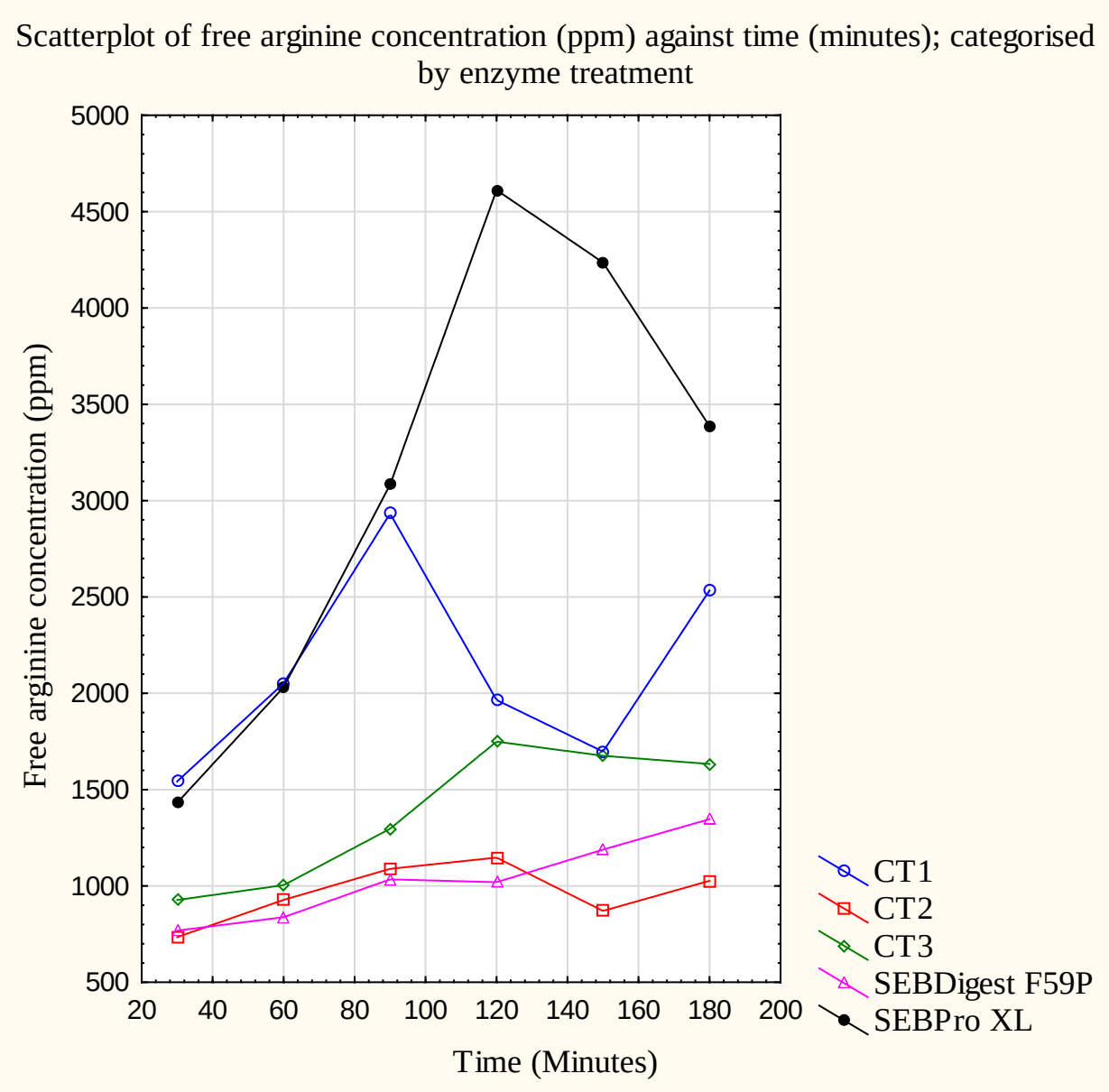

Figure 9-20; Progress curves for free arginine concentration with time for the five different enzyme treatments

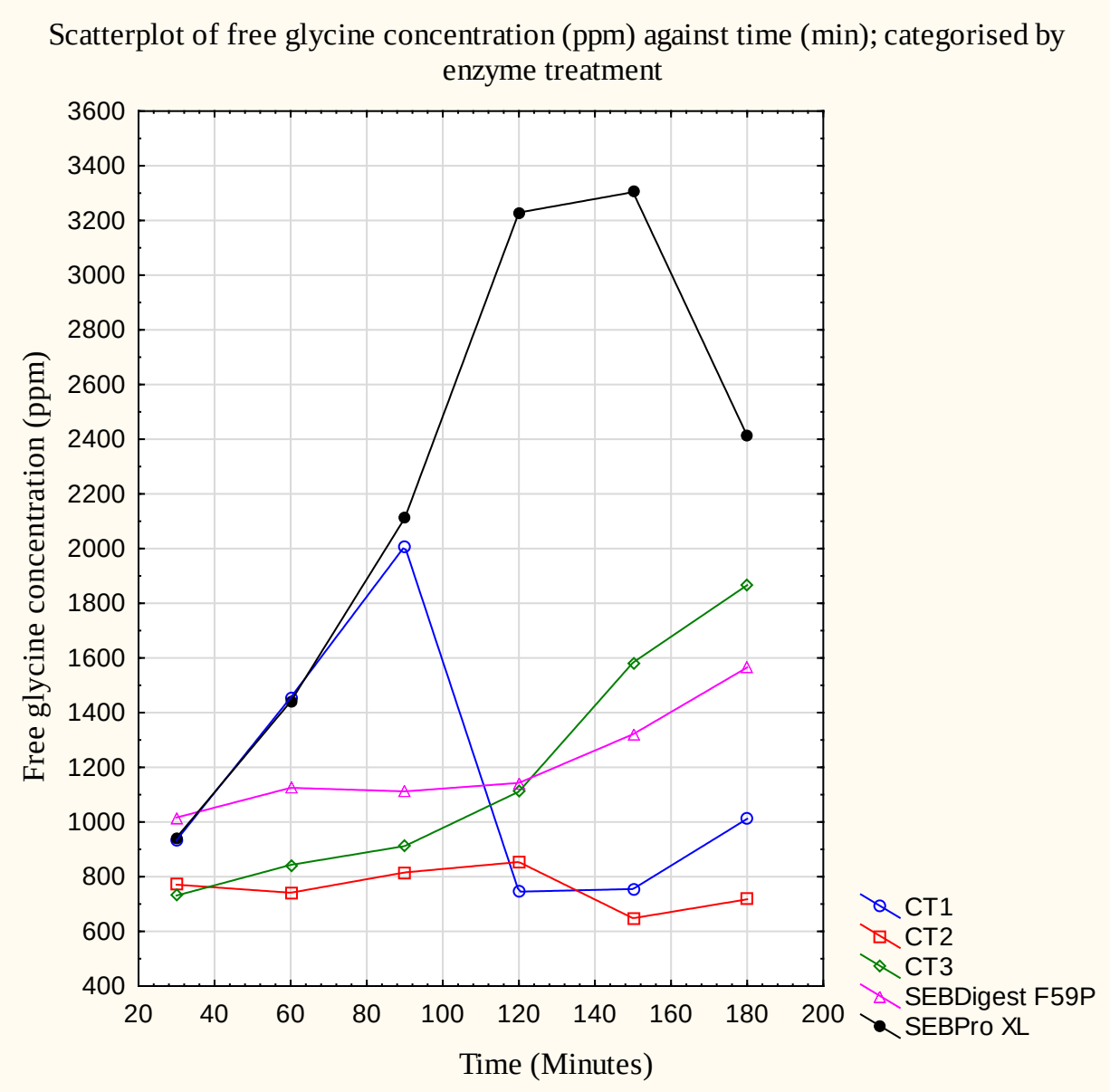

Figure 9-21; Progress curves for free glycine concentration with time for the five different enzyme treatments

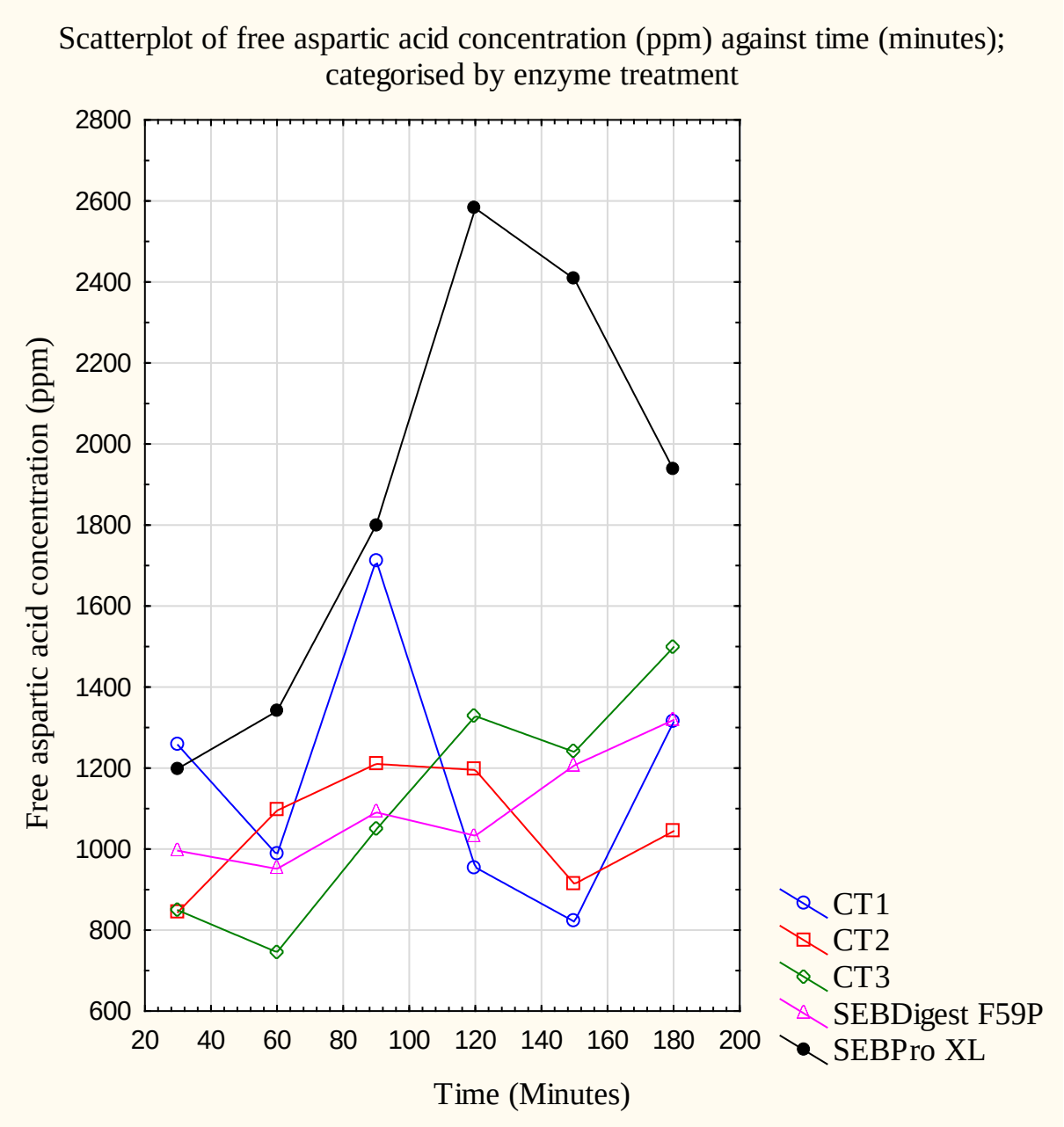

Figure 9-22; Progress curves for free aspartic acid concentration with time for the five different enzyme treatments

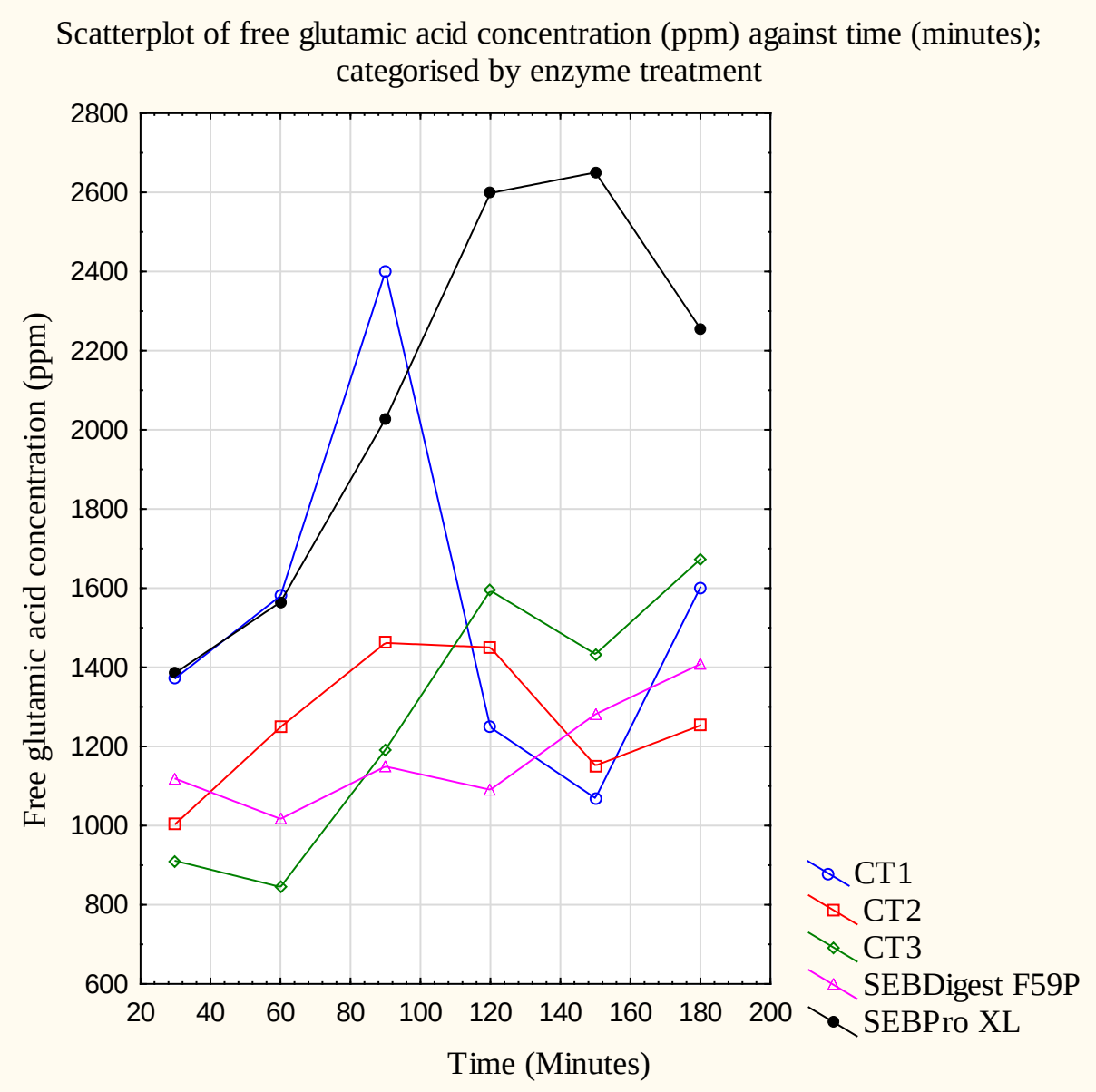

Figure 9-23; Progress curves for free glutamic acid concentration with time for the five different enzyme treatments

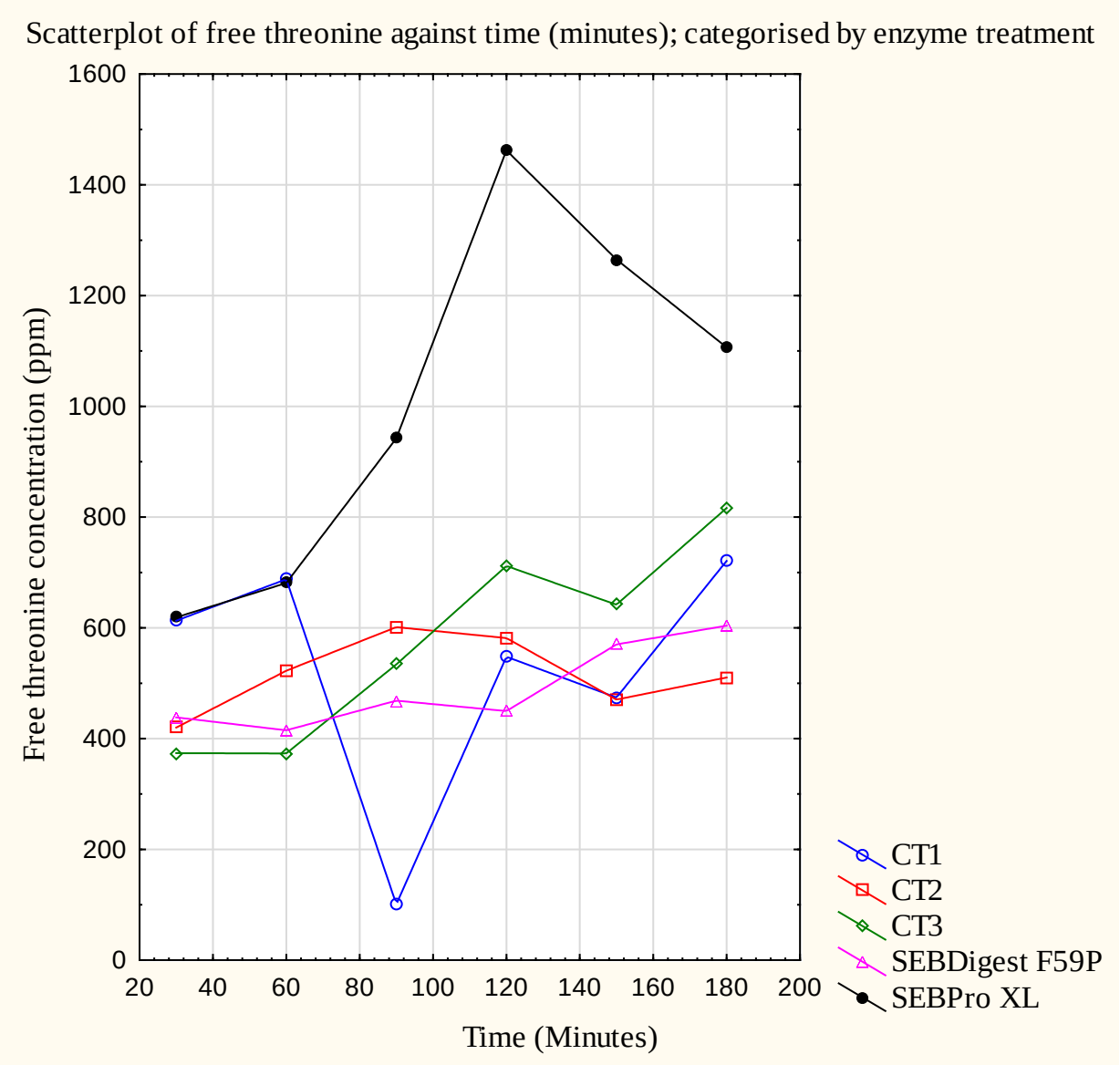

Figure 9-24; Progress curves for free threonine concentration with time for the five different enzyme treatments

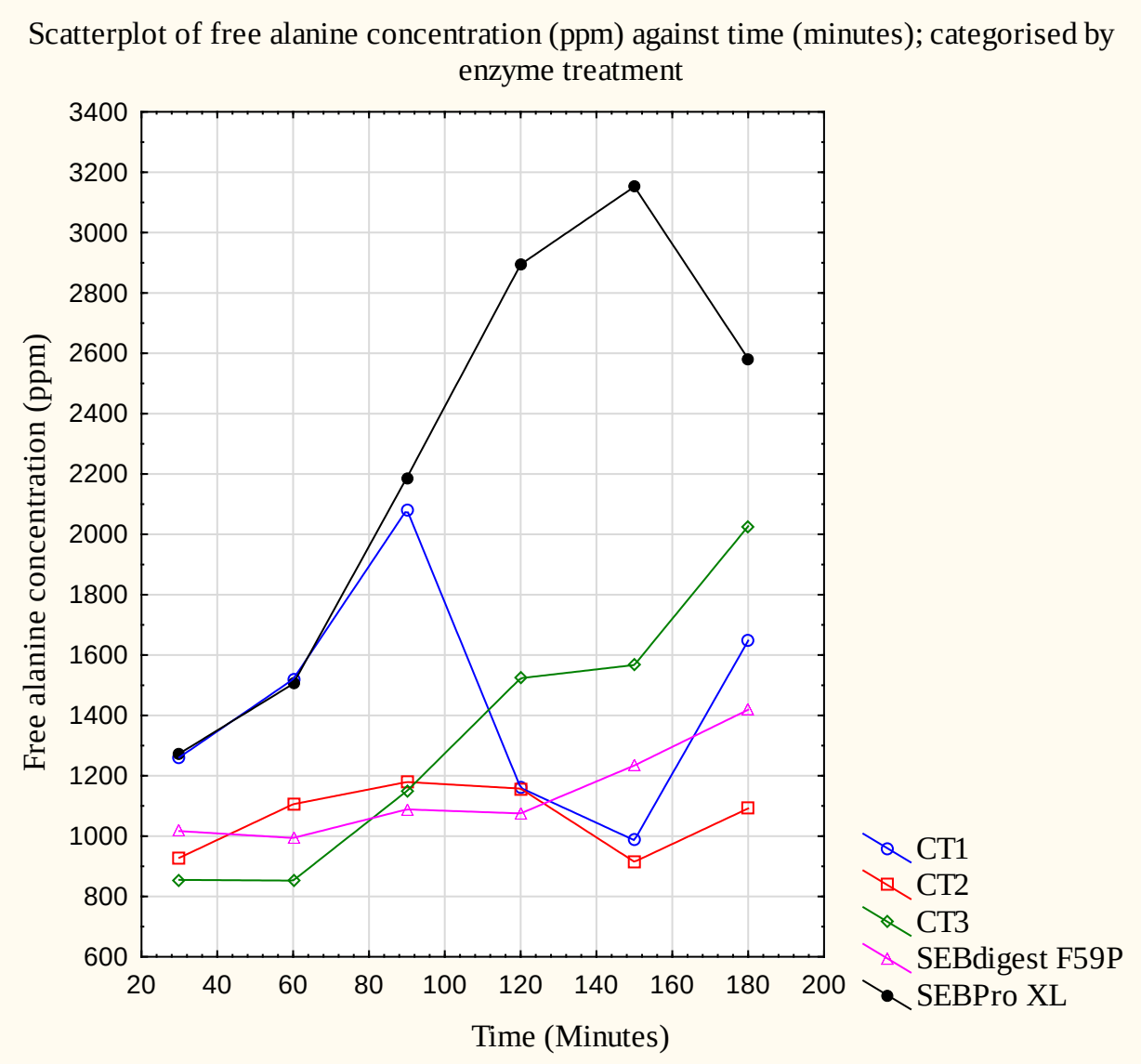

Figure 9-25; Progress curves for free alanine concentration with time for the five different enzyme treatments

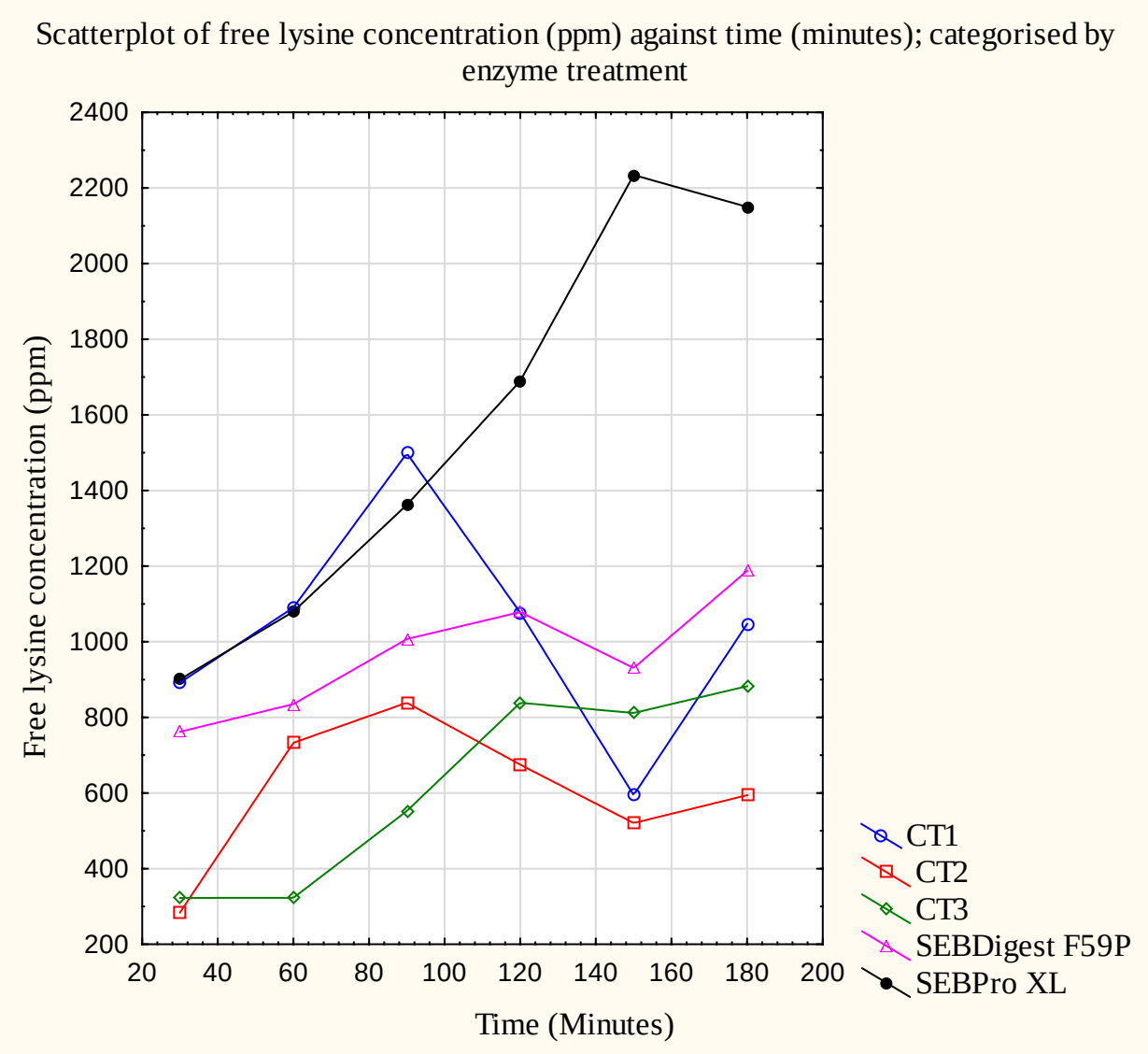

Figure 9-26; Progress curves for free lysine concentration with time for the five different enzyme treatments

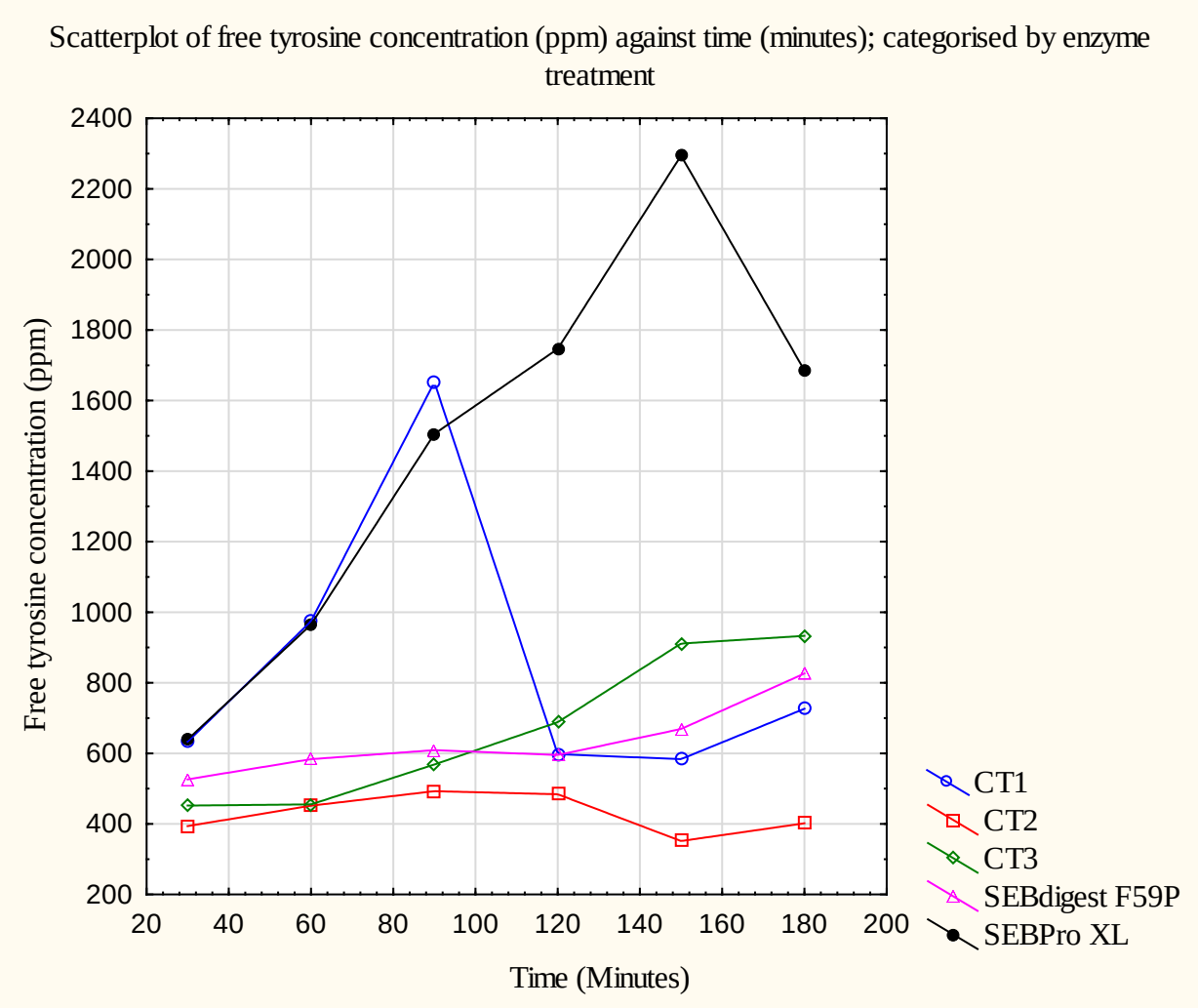

Figure 9-27; Progress curves for free tyrosine concentration with time for the five different enzyme treatments

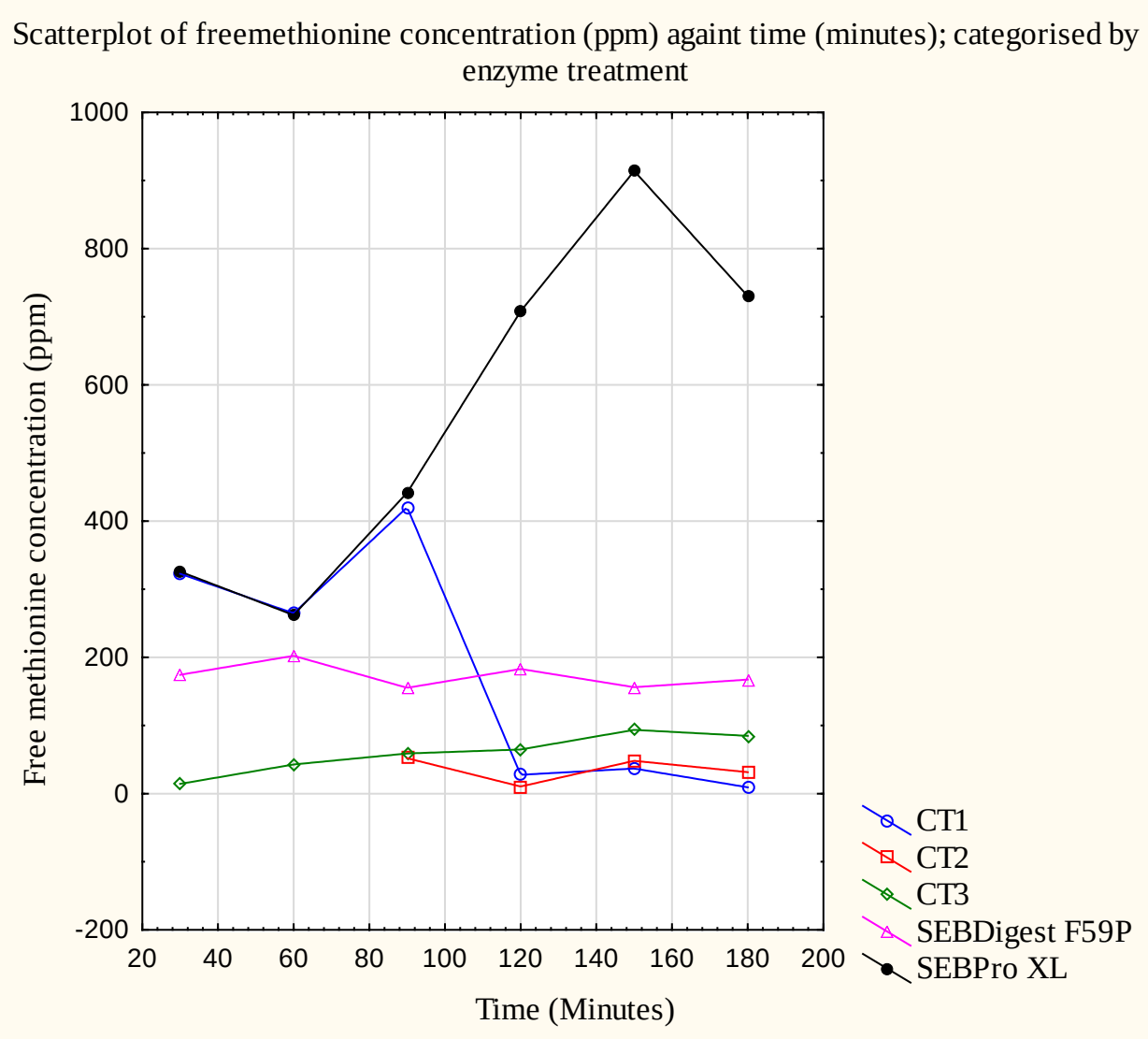

Figure 9-28; Progress curves for free methionine concentration with time for the five different enzyme treatments

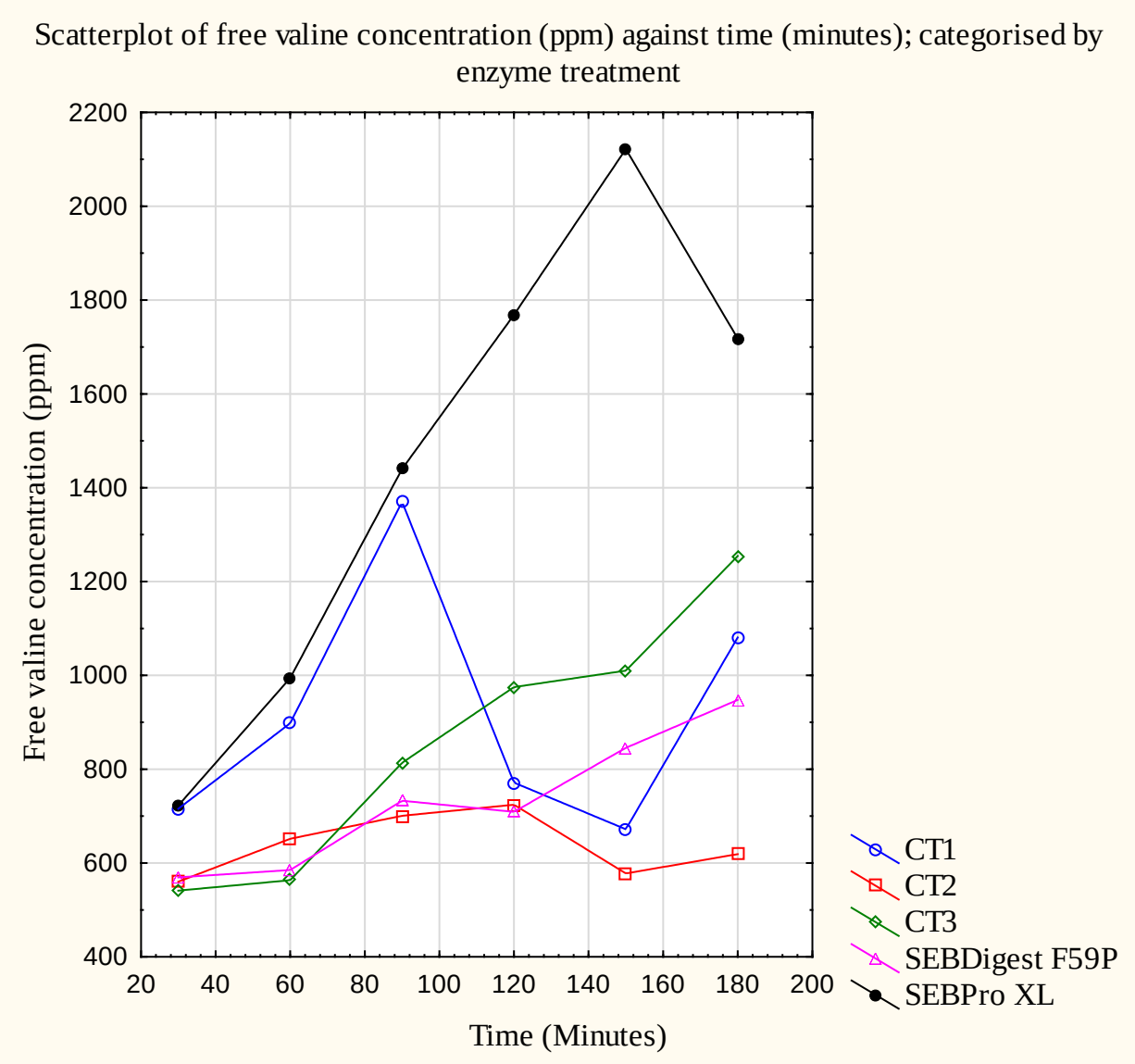

Figure 9-29; Progress curves for free valine concentration with time for the five different enzyme treatments

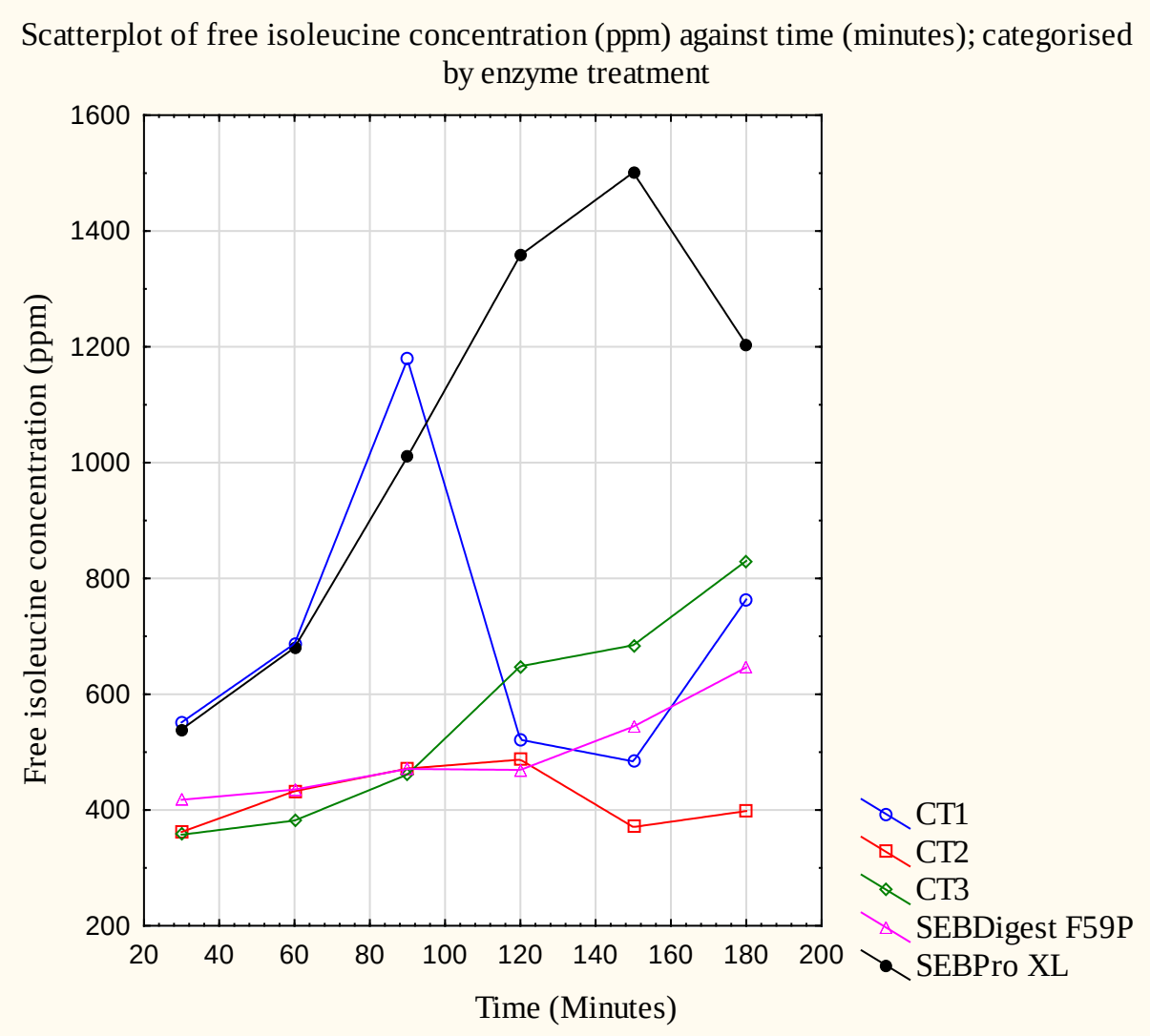

Figure 9-30; Progress curves for free isoleucine concentration with time for the five different enzyme treatments

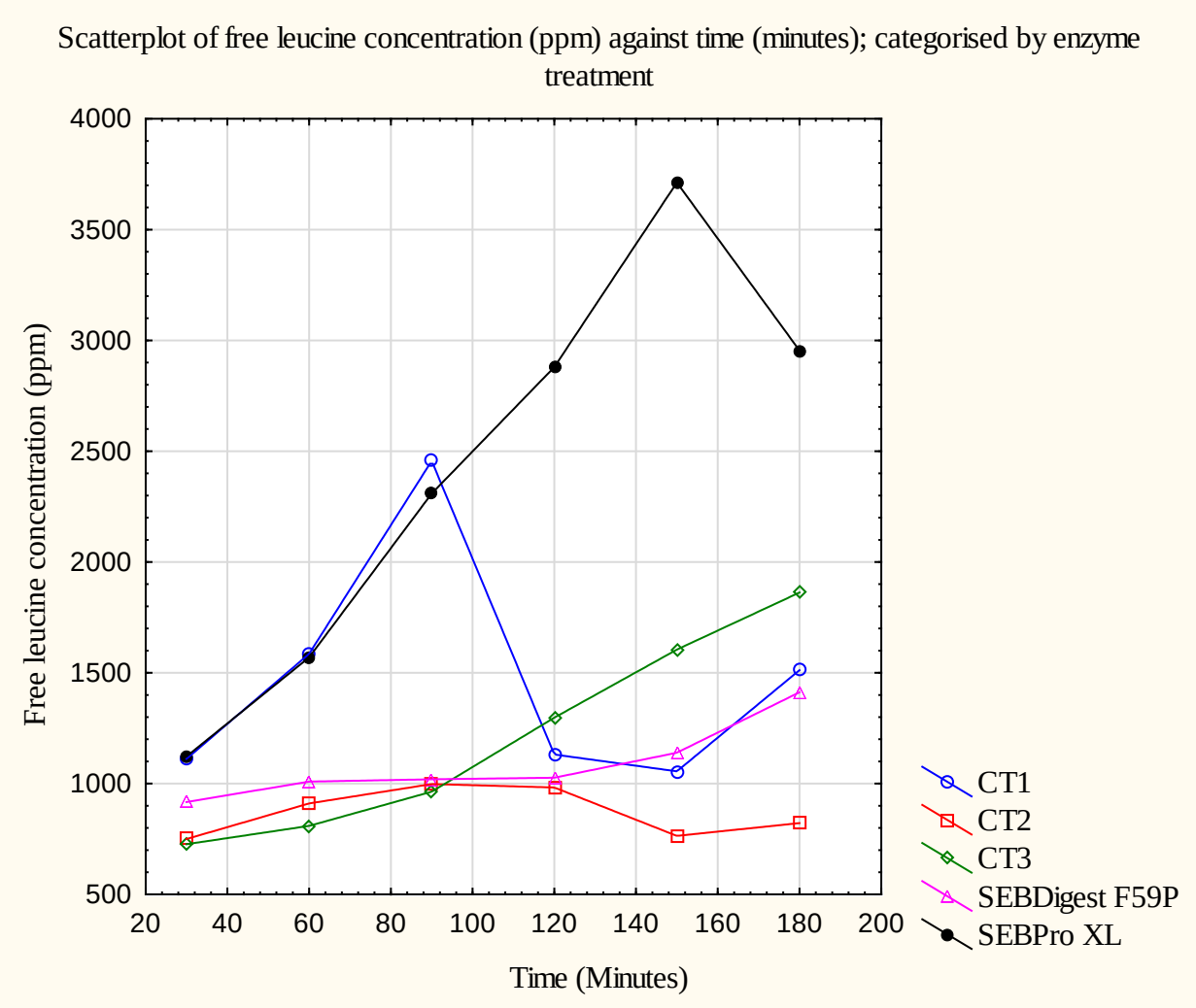

Figure 9-31; Progress curves for free leucine concentration with time for the five different enzyme treatments

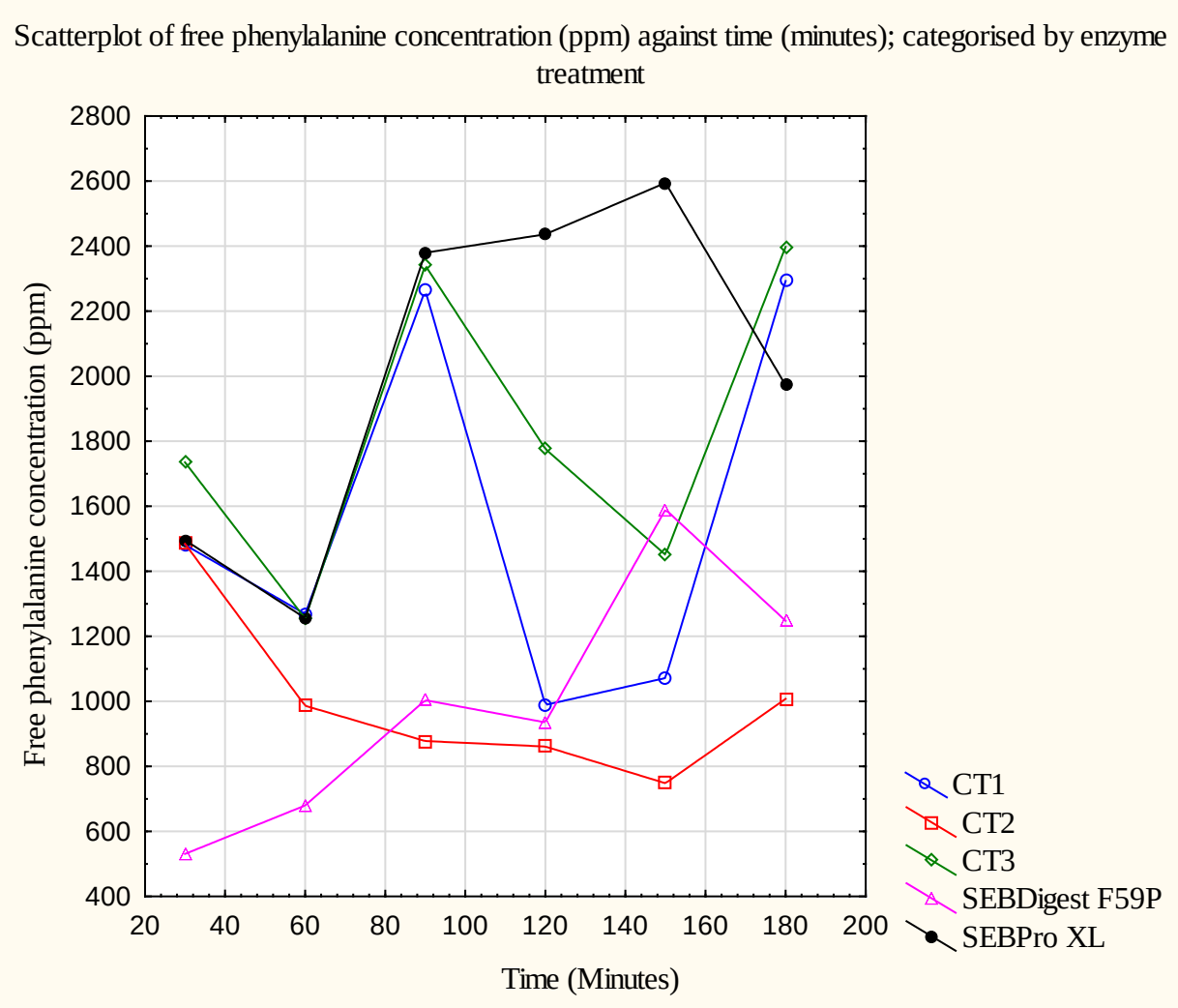

Figure 9-32; Progress curves for free phenylalanine concentration with time for the five different enzyme treatments

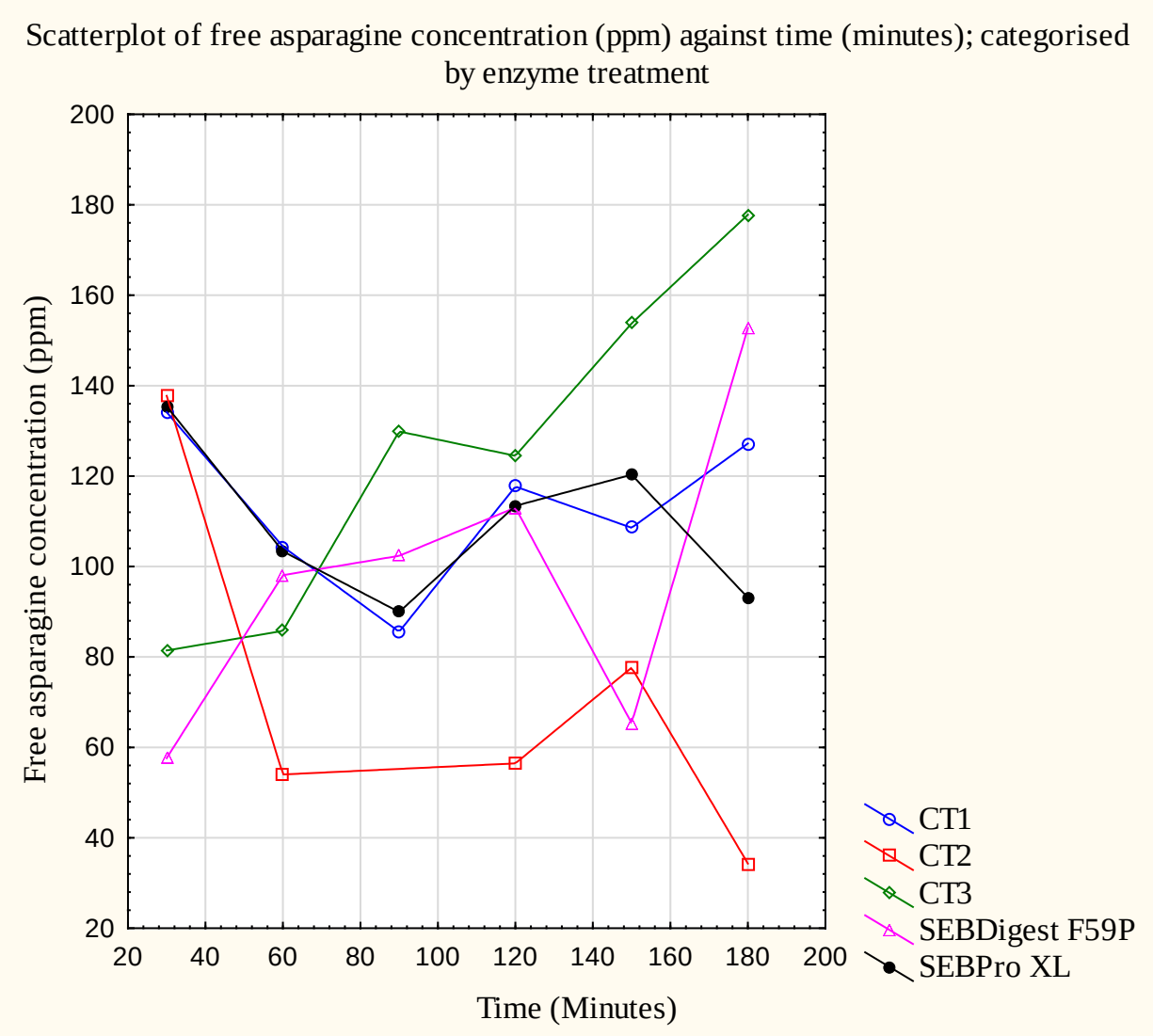

Figure 9-33; Progress curves for free asparagine concentration with time for the five different enzyme treatments

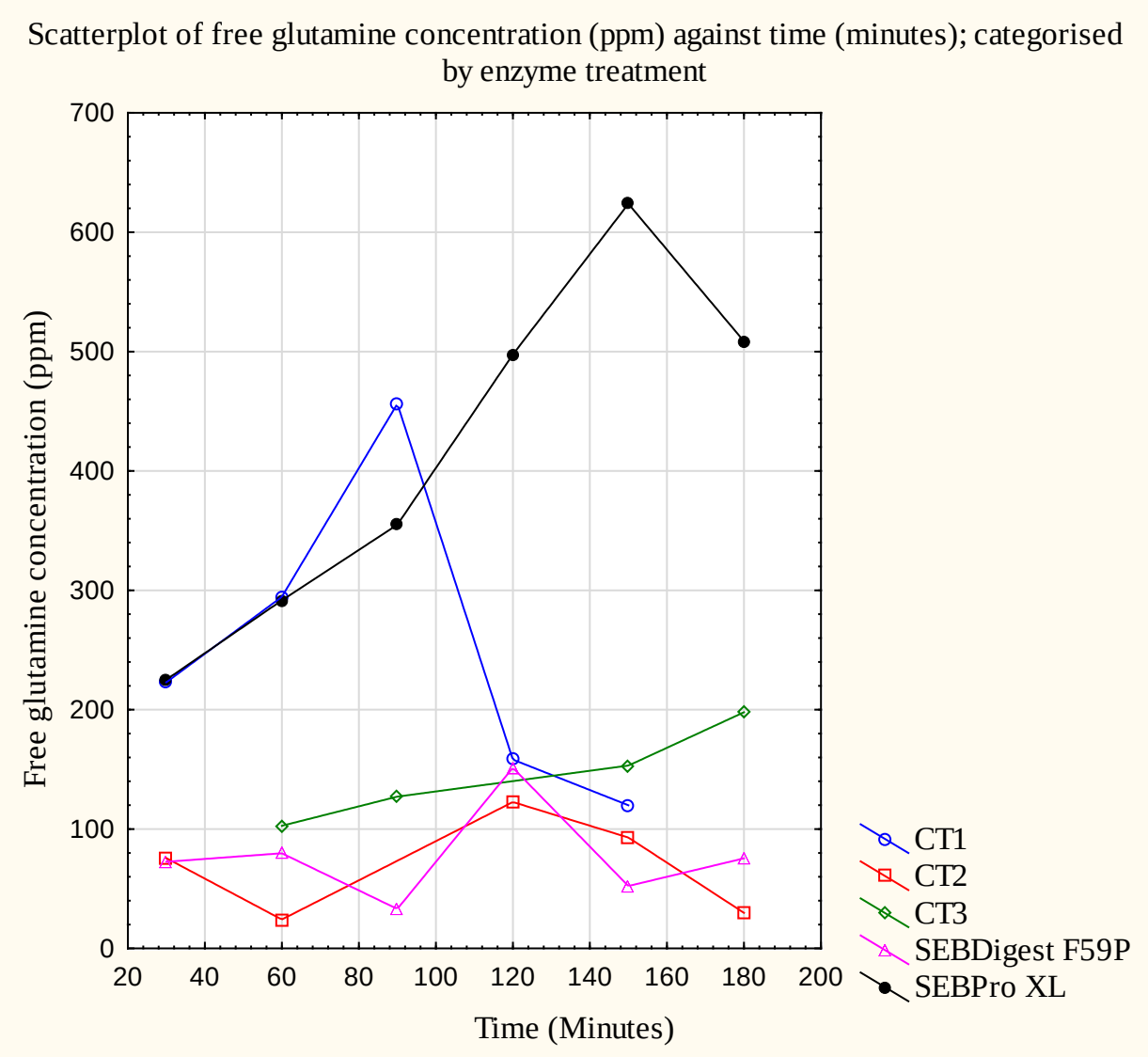

Figure 9-34; Progress curves for free glutamine concentration with time for the five different enzyme treatments

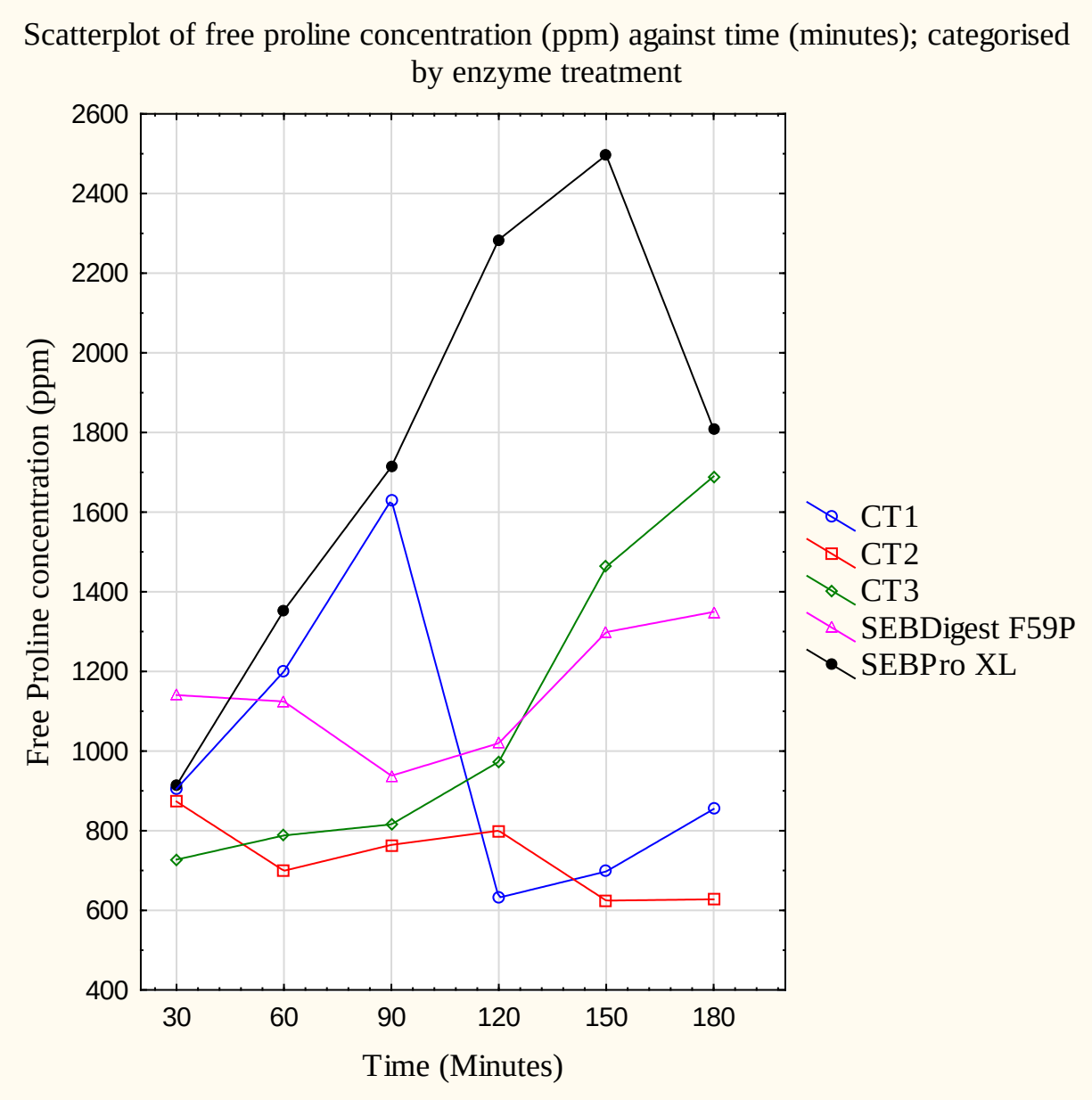

Figure 9-35; Progress curves for free proline concentration with time for the five different enzyme treatments